Steps toward nation-wide monitoring of non-indigenous species in Danish marine waters under the Marine Strategy Framework Directive

Andersen, Jesper H.; Møller, Peter Rask; Kallenbach, Emilie; Hesselsøe, Martin; Knudsen, Steen Wilhelm; Bekkevold, Dorte; Hansen, Brian Klitgaard; Thaulow, Jens

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DTU

Natural History Museum of Denmark
University of Copenhagen

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NIVA Denmark Water Research
– a subsidiary of the Norwegian Institute for Water Research

REPORT

Main Office
Gaustadalléen 21
NO-0349 Oslo, Norway
Phone (47) 22 18 51 00
Telefax (47) 22 18 52 00
Internet: www.niva.no

NIVA Region South
Jon Lilletuns vei 3
NO-4879 Grimstad, Norway
Phone (47) 22 18 51 00
Telefax (47) 37 04 45 13

NIVA Region East
Sandvikaveien 59
NO-2312 Ottestad, Norway
Phone (47) 22 18 51 00
Telefax (47) 62 57 66 53

NIVA Region West
Thormøhlens gate 53 D
NO-5006 Bergen Norway
Phone (47) 22 18 51 00
Telefax (47) 55 31 22 14

NIVA Denmark
Ørestads Boulevard 73
2300 Copenhagen S
Phose (45) 88 96 96 70
www.niva-danmark.dk

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Abstract

This report is the outcome of MONIS 2 – or in full, "Monitoring of Non-Indigenous Species in Danish Marine Water, phase 2" – and includes three deliverable: (1) a national Target Species List including 50 species, (2) a draft Technical Guidance Report, and (3) *in silico* designed and tested primers and probes for 48 of the 50 species on the Target Species List. The list is based on discussions at a workshop and subsequent scoring and ranking of relevant species. The draft Technical Guidance report is anchored in existing Standard Operating Procedures (i.e. protocols for sampling, storage and analysis) and adapted to the requirements of the Danish NOVANA programme. In addition, the report includes suggestions for next steps to take to implement and improve monitoring and assessment activities in regard to non-indigenous species in Danish marine waters.

4 keywords, Danish	4 keywords, English
1. Havstrategidirektivet	1. Marine Strategy Framework Directive (MSFD)
2. Ikke-hjemmehørende arter	2. Non-indigenous species (NIS)
3. Overvågning	3. Monitoring
4. eDNA-baserede metoder	4. eDNA based methods

Chief Scientist
Jesper Andersen

Research Director
Nikolai Friberg

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MONIS phase 2

Steps toward nation-wide monitoring of non-indigenous species in Danish marine waters under the Marine Strategy Framework Directive

Client: Danish Nature Agency

Preface

We report the outcome of the project "Monitoring of Non-Indigenous Species in Danish Marine Waters, phase 2" (MONIS 2), which has been initiated and funded by the Danish Nature Agency. The report follows up on MONIS 1, which is published in 2014 by the Danish Nature Agency:

- Andersen, J.H., S.A. Pedersen, J. Thaulow, F. Stuer-Lauridsen & S. Cochrane (2014): Monitoring of non-indigenous species in Danish marine waters. Background and proposals for a monitoring strategy and a monitoring network. Danish Nature Agency. 55 pp.

Three key deliverables included in the report:

- A national Target Species List including 50 species,
- A draft Technical Guidance Report describing in detail how to carry out sampling, storage and analyses, and
- Species-specific *in silico* designed and tested primers- and probe systems for 48 of the 50 species on the Target Species List. All systems have been matched and compared with nucleotide sequences available on the NCBI GenBank database.

In addition to the above, we propose next steps which are meant as a road map for the implementation of national monitoring and assessment activities in regard to Descriptor 2 specific requirements (i.e. non-indigenous species; NIS) under the EU Marine Strategy Framework Directive (MSFD).

The authors would like to thank Henrik Carl, Natural History Museum Denmark for permission to use the photo on the front cover as well as Norman Green for a critical review of an earlier version of this report.

Copenhagen, 7 April 2016

Jesper H. Andersen

Contents

Summary

The project "Monitoring of Non-Indigenous Species in Danish Marine Waters, phase 2" (MONIS 2) has been initiated and funded by the Danish Nature Agency in order to follow up on the proposed monitoring strategy and tentatively outlined design of a national monitoring programme targeting non-indigenous species in Danish marine waters. The monitoring activities are anchored in requirement in the EU Marine Strategy Framework Directive (MSFD), specifically in regard to Descriptor 2 concerning non-indigenous species (D2).

In order to make proposals suggested by MONIS 1 operational work has focused on three issues:

1. development of a national MSFD D2-specific Target Species List including 50 species,
2. development and writing of a draft technical Guidance Report based on the existing National Monitoring and Assessment Programme for the Aquatic and Terrestrial Environments (NOVANA) principles and structure for such reports, and
3. *in silico* (i.e. computer-based analysis) to compare nucleotide sequences for the upcoming development of primers for as many of the species on the Targets Species List as possible.

The Target Species List, the *in silico* developed primers and the Technical Guidance Report are included in this report. In combination, they represent important building blocks towards the initiation of nation-wide sampling covering both coastal waters and open marine areas in late spring / early summer 2016.

The process and strategy outlined by the MONIS 1 report can turn into a more practical phase where a Danish MSFD D2-specific monitoring programme can now be implemented.

1. Introduction

Danish marine waters are impacted by multiple human stressors, e.g. nutrient inputs (Ærtebjerg *et al.* 2003, Andersen & Conley 2009), fishing activities (Korpinen *et al.* 2012, Andersen & Stock (eds.) 2013), inputs of hazardous substances (Dahlöf & Andersen 2009), as well as physical modification (Andersen *et al.* 2012). The ecosystem health of Danish marine waters is accordingly assessed as being impaired (Naturstyrelsen 2012), especially in regard to eutrophication (Riemann et al 2015, Andersen *et al.* 2015), hazardous substances (Andersen *et al.* 2016), biodiversity (Andersen *et al.* 2014a) as well as 'ecosystem health' (Naturstyrelsen 2012).

Other human activities might also be important, e.g. introductions of non-indigenous species, but there is currently only scarce information about introduction and occurrence of non-native species in Danish marine waters (e.g. Stæhr & Thomsen 2012, Azour *et al.* 2015).

However, the EU Marine Strategy Framework Directive (MSFD) call for an ecosystem-based approach to the management of all European marine waters (Anon. 2008) and consequently Denmark, as well as all EU Member States with a coastline, are required to carry out so-called 'Initial Assessment' of pressures and environmental status and to implement specific monitoring activities.

The assessment in regard to 'good environmental status' (GES), being an overarching target to achieve in a MSFD-context, is nested, and the groups of indicators addressed are termed Descriptors. In total there are 11 descriptors and the GES definition for descriptor 2 (D2) is "Non-indigenous species introduced by human activities do not adversely alter the ecosystem" (Anon. 2010).

In Denmark, the Nature Agency has initiated a process aiming at a formulation of both a strategy for MSFD D2 monitoring as well as an interim proposal for a monitoring programme. This was done within the MONIS project (Andersen *et al.* 2014b). In order to follow up on this strategy and proposal for a monitoring programme, the Nature Agency published a tender requesting three key products:

1. the development of a national MSFD D2-specific Target Species List,
2. production of a draft Technical Guidance Report describing in details how to carry out sampling, storage and lab analyses, and
3. *in silico* development and testing of as many species-specific primer-probe assays as possible with regard to the national MSFD D2 Target Species List.

The tender was won by a partnership comprising NIVA Denmark Water Research, AmphiConsult, DTU Aqua and the University of Copenhagen/National Natural History Museum of Denmark as well as an associate partner being Eurofins Miljø AS.

In addition to the above three key products, the report also includes a range of suggestions for the next steps to arrive at an operational MSFD D2-specific monitoring programme.

2. What did we do?

The processes, principles and methods used for (1) development of a national MSFD D2-specific Target Species List, (2) drafting of a Technical Guidance Report, and (3) *in silico* development and testing of as many species-specific primer-probe assays as possible, are described in the following sections.

2.1 Towards a national MSFD D2 Target Species List

The ranking of marine species for a national MSFD D2 target list is based on different authorities' registrations and listing of non-indigenous and/or invasive species in the Danish marine areas (Naturstyrelsen 2008a, 2008b, Stæhr & Thomsen 2012) or within the boundaries of European seas (HELCOM 2015, OSPAR 2015). Additionally, two other lists established by Jensen (2013) and Møller (2015) were included in the making of the target species list, in order to fill in the blanks. As it appears from the overall list, the different lists are in accordance with one another.

Table 1: Summary of the species lists on which the national Target Species List is based.

Full reference
1. Berggren, U. (*pers. comm.*): Draft short list of marine non-indigenous species.
2. HELCOM (2015): List of Target Species currently in use in the HELCOM area. 1 pp.
3. Jensen, K. (2013): Target Species List 1 (established species) and Target Species List 2 (alert list). 2 pp.
4. Møller, P.R. (*pers. comm.*): Supplerende liste over marine fisk. (In Danish)
5. Naturstyrelsen (2008a): Sortlisten. Akvatiske arter. 1 pp.
6. Naturstyrelsen (2008b): Observationslisten. Akvatiske arter. 1 pp.
7. OSPAR (2015): OSPAR Target Species List (OSPAR Agreement 2015-10). 2 pp.
8. Stæhr, P.A. & Thomsen, M.S. (2012): Opgørelse over rumlig udbredelse, tidslig udvikling og tæthed af ikke-hjemmehørende arter i danske farvande. Fagligt notat fra DCE - Nationalt Center for Miljø og Energi. 14 pp.
9. Jensen, K. (*pers. comm.*)

Ranking of the target species is based on the following five criteria:

1. established non-indigenous species (NIS),
2. potential NIS,
3. invasiveness,
4. ease of determination by conventional methods, and
5. ease of determination by eDNA techniques.

These criteria are described in the following sections:

- **Criterion 1 – Established NIS**: The scores are based on the list of established species in Table 1. If a species is registered on one of the lists, it gets the score 8, whereas it is assigned the score 10, if it is appears on two lists (Jensen, K. (pers. Comm.) and Stæhr & Thomsen (2012)).If it is not registered on any of those two, it gets the score 0, and is considered as not yet established in Denmark (cf. Table 1).

- **Criterion 2 – Potential NIS**: If not yet established, a species' potential invasiveness is assessed. This assessment is rooted in the lists from OSPAR (2015) and HELCOM (2015). OSPAR and HELCOM cover the North-East Atlantic and Baltic Sea respectively and thereby indicate the largest dispersal areas for this study. For each of the lists the species occurs on, the species gets one point. In additional, a score from 1 to 3 is given if the species is mentioned in the Jensen (2013) List 2 of not yet established ("alert") species depending on the score given them. The Jensen score is derived from an appraisal of the potential for being invasive. Furthermore, if present on EU's regulation list (Berggren *pers. comm.*), it gets the score 3.

- **Criterion 3 – Invasiveness**: The third criterion describes the degree of invasiveness a species is assessed to have. The scores 2, 4, 6 can be obtained, with 6 being the most invasive. The scores are based on a literature study, on the species effect on: 1) colonization of conservational valuable habitats, 2) impact on native species, 3) effect on ecosystem functioning and 4) economical as well as public health effect (Madsen *et al.* 2014). Furthermore, if literature was not available, expert judgements was applied.

- **Criterion 4 – Ease of determination**: The score given under this criterion is based on knowledge of the degree of difficulty in the characterization of the species. If the species is easy to confuse with similar looking species, it received the score of 1, whereas, if it is very characteristic or peculiar and hence, easy to identify, it received the score of 3.

- **Criterion 5 – Ease of determination by environmental DNA (eDNA)**: Different species exhibit different levels of complexity in regard to determination with eDNA-based methods (see Díaz-Ferguson *et al.* 2014, Goldberg *et al.* 2015, Taberlet *et al.* 2012, Thomsen & Willerslev 2015, Thomsen *et al.* 2012, Wilcox *et al.* 2013). Species with many sympatric species generally more difficult to determine with eDNA methods than species with no or few close relatives. The ease of determination by eDNA also depends on the availability of DNA reference sequences in public data facilities (e.g. National Center for Biotechnical Information (NCBI), The Barcoding Of Life Data System (BOLD)). The expert judgement of the ease of determination is the basis for the scoring of values between 1 and 3.

The above critera and the ranking process was planned early in th MONIS 2 process (see Appendix A). Each species is evaluated and scored in regard to these 5 criteria. Finally, the scores are summed together. The 51 species getting the highest scores are those included in the MSFD D2-specific Target Species List. The 37th ranked species, a parasite *Anguillicola crassus*, was excluded from the final list as the chances of detecting a parasite by using eDNA-based methods were anticipated to be low. The total number of species on the final list being passed on to further analysis thus came out at 50.

2.2 Technical Guidance Report for eDNA-based monitoring activities

A robust and scientifically credible Technical Guidance Report is needed to prevent both false positive (e.g. due to cross-contamination or non-specific primers) or false negative (e.g. due to technical error during sampling, filtration, extraction or non-amplifying primers) registration of species presence.

False positives and negatives are of special concern when estimating the potential detrimental effect of non-indigenous species. Large scale eDNA surveys conducted in the USA (Jerde *et al.* 2013) and Japan (Fukumoto *et al.* 2015) serve as good examples of the stringency of Standard Operating Procedures (SOPs) needed when multiple people are involved in sample collection and possibly also laboratory analyses. Thus, for documentation, the SOPs for every step in the Technical Adcisory TA have to be written with the utmost detail for the entire process from sampling to laboratory analysis.

The Techincal Guidance provided for this MONIS phase 2 report considers all 48-50 primer-probe assays systems reviewed as tentative. None of the systems have yet been tested on actual DNA extracts from target-species and sympatric non-target species (i.e. *in vitro* test). Without knowledge of how these systems perform on actual DNA, it is unknown whether any of the suggested systems in fact are able to provide true positive detection of eDNA from the target-species. It should be kept in mind that the *in silico* test only is based on nucleotide sequence comparison. Therefore, preliminary *in vitro* tests for each system that is developed is mandatory to estimate the specificity of each system, before any direct tests can be completed in environmental samples.

2.3 Development of an eDNA-based single-species detections system

An eDNA detection system was based on *in silico* development and testing using a polymerase chain reaction (PCR) detection system that is only based on available DNA-sequence information from target and non-target species.

We have focused on developing a 'single-species detection system' for each of the 50 species on the Target Species List. The 'single-species detection system' is based on the design of a species-specific primer-probe assay developed for quantitative PCR (qPCR) platforms. This approach will during applications circumvent the need for routinely sequencing of PCR products. In addition, species-specific qPCR assays may potentially enable future quantitative applications of eDNA, i.e. it may be possible to estimate the abundance of eDNA copies from a target species per volume of filtered water. This may enable correlations between the prevalence of eDNA with population assessments or other conventional monitoring data. Also, species-specific qPCR systems are considered more sensitive when targeting eDNA fragments with low abundance and this will reduce the risk of false negatives.

Contrary to the invertebrate and vertebrate species on the Target Species List, phytoplankton and macro algae have until now received relative moderate attention in regards to eDNA or direct species identification from filtrated water samples.

For each of the species on the Target Species List relevant mitochondrial DNA (e.g. CytB, CO1) or nuclear sequences (e.g. 28S) were identified via the online NCBI database (www.ncbi.nlm.nih.gov) and aligned using e.g. the BioEdit software, with the same sequence for closely related species or other species showing sequence comparison. Primers and probes were positioned in sequence sections where the difference between the target and non-target species was the largest. Once positioned, each assay was screened for unknown compatibility in other non-target species by conducting a nucleotide BLAST search via the online NCBI database. If non-related, non-target species were identified during this screening, the primer-probe assay was re-designed to improve the species-specification of the assay. Along with the sequence information, the degree of base pair differentiation for a representative number of non-target species is given. This was done to indicate the likelihood that the individual primers and the probe would not amplify a non-target species.

3. Results

The results in regard to (1) development of a national MSFD D2-specific Target Species List, (2) drafting of a Technical Guidance Report, and (3) *in silico* development of as many species-specific primers as possible, are described in the following sections.

3.1 Target Species List

By using the method described above, a species list of 50 species was deduced (Table 2). In total 21 invertebrates, 12 fish, 12 species of submerged aquatic vegetation, and 5 species of phytoplankton appears on the list. With the exception of 4 species, all of the 50 species, which constitute the Non-indigenous target species list, are established in Denmark.

The MONIS 2 draft Target Species List can be found in Table 2 with supplementary information in Appendix D.

3.2 Technical Guidance Report

A draft Technical Guidance Report has been written in Danish with the tentative title: "Teknisk Anvisning for indsamling af marine vandprøver og analyse for eDNA (*environmental* DNA)" and focuses on the following elements:

1. Sampling and filtration of water
2. Preservation of filters
3. Storage of filters
4. Extraction of DNA
5. Species-specific eDNA detection using qPCR

For details, please confer with the draft text in Appendix B.

3.3 A first generation of MSFD D2-related primers

MONIS, phase 2 reports a total of 48 primer and probe assays out of the 50 species on the Species Target List. Details can be seen in Appendix D.

Table 2: Proposed Target Species List for monitoring of non-indigenous species in Danish marine waters.

No.	Group	Species
1.	Fish	*Neogobius melanostomus* (Pallas, 1814)
2.	Invertebrate	*Crassostrea gigas* (Thunberg, 1793)
3.	Invertebrate	*Crepidula fornicata* (Linnaeus, 1758)
4.	Invertebrate	*Teredo navalis* Linnaeus, 1758
5.	Phytoplankton	*Karenia mikimotoi* (Miyake & Kominami ex Oda, 1935) (G. Hansen & Moestrup, 2000)
6.	Fish	*Oncorhynchus mykiss* (Walbaum, 1792)
7.	Zooplankton	*Mnemiopsis leidyi* Agassiz, 1865
8.	Invertebrate	*Eriocheir sinensis* Milne-Edwards, 1853
9.	Invertebrate	*Rhithropanopeus harrisii* (Gould, 1841)
10.	Phytoplankton	*Prorocentrum minimum* (Pavillard) J. Schiller, 1933
11.	Subm. Veg.	*Gracilaria vermiculophylla* (Ohmi) Papenfuss, 1967
12.	Subm. Veg.	*Sargassum muticum* (Yendo) (Fensholt, 1955)
13.	Invertebrate	*Cordylophora caspia* (Pallas, 1771)
14.	Invertebrate	*Styela clava* Herdman, 1881
15.	Invertebrate	*Dreissena polymorpha* (Pallas, 1771)
16.	Fish	*Acipenser baerii* Brandt, 1869
17.	Fish	*Acipenser gueldenstaedtii* Brandt & Ratzeburg, 1833
18.	Fish	*Acipenser ruthenus* Linnaeus, 1758
19.	Fish	*Acipenser stellatus* Pallas, 1771
20.	Fish	*Huso huso* (Linnaeus, 1758)
21.	Fish	*Oncorhynchus gorbuscha* (Walbaum, 1792)
22.	Fish	*Salvelinus fontinalis* (Mitchill, 1814)
23.	Subm. Veg.	*Colpomenia peregrina* (Sauvageau, 1927)
24.	Invertebrate	*Ensis americanus* (Gould, 1870)
25.	Subm. Veg.	*Dasya baillouviana* (Gmelin) Montagne, 1841
26.	Subm. Veg.	*Heterosiphonia japonica* Yendo, 1920
27.	Subm. Veg.	*Spartina anglica* Hubbard
28.	Phytoplankton	*Heterosigma akashiwo* (Y.Hada) Y.Hada ex Y.Hara & M.Chihara, 1987
29.	Phytoplankton	*Pseudochattonella farcimen* (W. Eikrem, B. Edvardsen & J. Throndsen) W. Eichrem, 2009
30.	Invertebrate	*Molgula manhattensis* (de Kay, 1843)
31.	Zooplankton	*Cercopagis pengoi* (Ostroumov, 1891)
32.	Invertebrate	*Homarus americanus* Milne-Edwards, 1837
33.	Invertebrate	*Paralithodes camtschaticus* (Tilesius, 1815)
34.	Invertebrate	*Didemnum vexillum* Romanov, 1989
35.	Subm. Veg.	*Fucus evanescens* C. Agardh, 1820
36.	Invertebrate	*Petricolaria pholadiformis* (Lamarck, 1818)
37.	Fish	*Perccottus glenii* Dybowski, 1877
38.	Invertebrate	*Elminius modestus* Darwin, 1854
39.	Invertebrate	*Ficopomatus enigmaticus* (Fauvel, 1923)
40.	Invertebrate	*Marenzelleria viridis* (Verrill, 1873)
41.	Invertebrate	*Ocenebra inornata* (Récluz, 1851)
42.	Invertebrate	*Potamopyrgus antipodarum* (Gray, 1843)
43.	Phytoplankton	*Pseudochattonella verriculosa* (Y.Hara & M.Chihara) S.Tanabe-Hosoi, D.Honda, S.Fukaya, Y.Inagaki & Y.Sako, 2007
44.	Subm. Veg.	*Codium fragile* subsp. *fragile* (Suringar) Hariot, 1889
45.	Fish	*Carassius auratus* (Linnaeus, 1758)
46.	Fish	*Cyprinus carpio* (Linnaeus, 1758)
47.	Invertebrate	*Mya arenaria* Linnaeus, 1758
48.	Zooplankton	*Penilia avirostris* Dana, 1849
49.	Invertebrate	*Diadumene lineata* (Verrill, 1870)
50.	Subm. Veg.	*Bonnemaisonia hamifera* Hariot, 1891

4. Discussion and recommendations

The methods used, the results and the suggested next steps in regard to (1) development of a national MSFD D2-specific Target Species List, (2) drafting of a Technical Guidance Report, and (3) *in silico* development and testing of as many species-specific primers as possible, are discussed in the following sections.

The proposed Target Species List (Table 2) is the first of its kind in a national context and based on the currently best available information. However, it should be regarded as a "living" document subject to regular updates as new information and regulation become available.

The draft Technical Guidance Report (Appendix B) is also the first of its kind in the context of the NOVANA programme; the descriptions of eDNA-based methods have so far not been included in NOVANA as a standard methodology. The Technical Guidance Report deals with sampling, preservation and laboratory analyses and does in our opinion open the door for sampling with a specific focus on MSFD D2.

Recommendation 1: The Technical Guidance Report should be discussed and tentatively approved for sampling and storage of filters as this would enable the initiation of sampling activities as soon as possible, but not later than summer of 2016.

As for the proposed Target Species List, the Technical Guidance Report should also be considered a "living" document and updated whenever relevant, e.g. based on acquired experience for the use of it or when methods are further developed and tested for operational monitoring and analyses.

Another reason for commencing the sampling activities is that it has been suggested to store all filters in a national Filter Archive as this – at a later stage – would provide a possibility to reanalyse filters focusing on a broader range of species than those treated here. However, for prolonged storage it is recommended to extract DNA from filters, and archive the extracted DNA. If sampling has already been initiated, it is extremely important that similar types of filters are used, and sampling and storage protocols are uniform to ensure samples are comparable across sampling locality and sampling time.

Recommendation 2: The Danish Nature Agency should liaise with a partner capable of running the suggested Filter Archive as this would secure proper handling of filters sampled in 2016 and onwards.

Some institutions that potentially could host the suggested Filter Archive identified by MONIS-2 are: Eurofins Miljø A/S or Aarhus University. The National History Museum in Denmark (NHMD) has officially been appointed as a national cryo-facility, but this is something that will be implemented and operational at a later stage.

All eDNA-based activities should be coordinated and directly linked to the monitoring based on conventional sampling methods, primarily in regard to phytoplankton, zooplankton, submerged aquatic vegetation, fish, seabirds and marine mammals. This coordination should take place as soon as possible and prior to the implementation of eDNA-based sampling.

Recommendation 3: It is strongly recommended to place the stations for sampling of eDNA in connection with stations or areas where conventional MSFD D2-targeted sampling is being ongoing or planned (see MONIS-1 report; Andersen *et al.* 2015). This will enable cross-comparisons and validation.

Following and implementing the above recommendations 1-3 will in our understanding be a critical first step towards an eDNA-based MSFD D2-specific monitoring programme in Denmark. Next steps should

include further development of primers and probe systems and must include *in vitro* testing of target- and non-target species in 2016 and 2017. This will ultimately lead to the development of reporting guidelines.

Recommendation 4: Initiation of a process leading to more and better primer-probe assays is a prerequisite for successful implementation of a nation-wide MSFD D2 monitoring.

In order to strengthen the basis for the proposed activities further and continuous development of primer-probe assays is required, especially for those species on the Target Species List that are not yet considered 'operational' (i.e., where species-specific primer-probe assays could not be designed). In addition, the *in silico*-based primer-probe assays in appendix C should be tested and evaluated in order to support prioritization in regard to the further development of primers. Before any of the 50 provided primer and probe systems can be utilized on environmental samples, initial *in vitro* test is needed. *In vitro* test includes tests on extracted DNA from target –and sympatric non-target species which must be performed to confirm the expected specificity of each system. Without *in vitro* test it is not possible to rule out false positive and false negative detection arising from any of the developed systems. *In vitro* testing requires a comprehensive cryo-archive of tissue samples collected from both target- and non-target species. The NHMD currently holds one of Denmark's largest collections of tissue samples from various organisms, and it is therefore recommended that the mandatory *in vitro* tests are initiated by the NHMD or in very close collaboration with the NHMD.

Finally, we suggest establishing an informal network in regard to the eDNA-based MSFD D2 monitoring activities in Danish marine waters. Key objective for this activity is to support the Danish Nature Agency in implementing the recommendation and result from the MONIS-1 and MONIS-2 projects and to provide science-based support in relation to marine eDNA-based monitoring activities.

Recommendation 5: It is recommended that the Danish Nature Agency establishes a national network or working group which can provide guidance and scientific support with regards to implementation of eDNA-based method in the NOVANA/MSFD-specific marine monitoring activities in Denmark.

In a longer perspective, the suggested Danish activities would need to be coordinated with more or less identical work in progress in neighbouring countries – primarily Sweden and Germany – in order to avoid duplicate effort and the waste of scarce resources.

Recommendation 6: It is strongly recommended that Danish Nature Agency informs neighbouring countries and relevant Regional Marine Conventions about the Danish progress in establishing an eDNA-based monitoring network and in a longer perspective to coordinate the development of specific primers (*inter alia*, e.g. including cost-sharing).

Based on the work carried out, the following conclusions can be drawn:

1. Sampling activities can in principle – pending tentative approval of the MONIS-2 Technical Guidance Report and the initiation of a Filter Archive – be planned and implemented from mid-summer of 2017.
2. A further testing and development of primer-probe assays is required and should be carried out in 2016 and 2017. This testing should include *in vitro* testing of tissue extracted DNA from target and non-target species.
3. A network or working group should be established to provide guidance and scientific support for the Danish Nature Agency and the implementation of eDNA-based methods in the national marine monitoring programmes and activities.

If any of the above conclusions are not being followed up, the forthcoming 2018 MSFD Initial Assessment may lack scientific credibility and be in conflict with pan-European guiding principles for the assessment and reporting of the MSFD.

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Appendix A: Minutes of the TSL workshop

Dato:	Fredag den 18. september 2015	
Sted:	NIVA Danmark, Ørestand Boulevard 73, 2300 København S	
Deltagere:	Jesper H. Andersen	NIVA Danmark
	Per Andersen	Orbicon
	Dorte Bekkevold	DTU Aqua
	Ulrik Berggren	Naturstyrelsen
	Stine Christiansen	Naturstyrelsen
	Nikolai Friberg	NIVA Danmark
	Martin Hesselsøe	Amphi Consult
	Kathe R. Jensen	Statens Naturhistoriske Museum
	Steen W. Knudsen	Statens Naturhistoriske Museum
	Peter B. Mortensen	Eurofins
	Peter Rask Møller	Statens Naturhistoriske Museum
	Peter Stæhr	DCE-AU
	Hans Erik Svart	Naturstyrelsen
	Jens Thaulow	NIVA Danmark
	Mary Wisz	DTU Aqua

1. Velkomst, introduktion og formål

1.1 Nikolai Friberg og Jesper Andersen indledte med at byde velkommen til workshoppen og til NIVA Danmark. MONIS 1-projektet og baggrunden for MONIS 2-projektet blev kort introduceret.

1.2 Samtlige deltager præsenterede sig via en tour-de-table.

1.3 Det blev understreget, at formålet med workshoppen, som er en del af MONIS 2-projektet, var at at drøfte processen og evt. kriterier for udarbejdelse af en dansk MSFD D2 Target Species List, som skal lægges til grund for udviklingen af op til 50 in silico-testede primers.

2. MSFD og overvågning af NIS i danske farvande

2.1 Naturstyrelsen, ved Ulrik Berggren og Hans Erik Svart, gjorde indledningsvis rede for en række forvaltningsmæssige forhold af betydning for den fremtidige nationale overvågning af ikke-hjemmehørende arter i de danske farvande, herunder EU-direktiver og –forordninger, regionale konventioner (HELCOM og OSPAR) og en række nationale tiltag m.v.

2.2 Naturstyrelsen lagde vægt på at udarbejdelsen af en antional MSFD D2 Target Species List tager udgangspunkt i eksisterende lister (internationale og nationale), herunder at der overvejende fokuseres på arter, som allerede er tilstede i de danske farvande eller hvor der er international krav til overvågning, fx. EU-forordningen.

2.3 Naturstyrelsen gjorde det klart at den af MONIS 2 udarbejdede Target Speciel List vil være et første bud på hvilke arter, som skal overvåges samt være et levende dokument som fra tid til anden og efter behov skal opdateres.

2.4 Styrelsen lagde vægt på forventningsafstemning og gjorde i den forbindelse følgende klart: Styrelsen forventer, (1) at en kriterie-baseret dansk MSFD D2 Target Species List bliver udarbejdet som en del af MONIS 2-projektet, (2) at kriterierne tager udgangspunkt i tilstedeværelse, risisci og (3) om hvorvidt de pågældende arter er relvante i en eDNA-sammenhæng. Succeskriteriet er ikke at der nødvendigvis at der bliver udviklet primere for 40-50 arter på listen, men at der blandt de arter, som kommer på, er så mange som muligt der med sikkerhed kan identificeres til art med eDNA.

2.5 Denne vigtige udmelding fra styrelsen blive kort drøftet. Der var blandt MONIS 2-partnerne stort tilfredshed med denne udmelding, specielt styrelsens tilkendegivelse af at succeskriteriet for MONIS 2 er operationalitet frem for et bestemt antalt primere.

3. Amphi Consult og eDNA: Erfaringer fra feskvandsovervågningen. Hvor er vi nu og hvor går vi hen?

3.1 Martin Hesselsøe orienterede kort om Amphi Consults arbejde, specielt i relation til eDNA og projekterne vedrørende overvågning af udvalgte arter i ferskvand. Det blev herunder understreget, at MONIS 2 alene kan bidrage til udviklingen af et single species detection system.

4. DTU Aqua og eDNA-baseret overvågning m.v. af fisk

4.1 Dorte Bekkevold orienterede kort om DTU Aquas arbejde med eDNA, specielt i forhold til kommercielle fiskearter og en række af de problemstillinger, der kan knytte sig hertil, bl.a. sæsonvariationer

5. Det nationale Fiskeatlas-projekt og andre relevante SNM-aktiviteter

5.1 Peter Rask Møller orienterede kort om SNM's arbejde med overvågning af kystnære fisk, herunder det nationale Fiskeatlas-projekt m.v. I forlængelse heraf orineterede Sten W. Knudsen kort om SNM's arbejde med eDNA i forhold til udvalgte fiskearter, herunder en række at de udfordringer i forbindelse med metodikken som man fagligt må forholde sig til.

6. Første skridt mod en dansk MSFD D2 Target Species List

6.1 Indledningsvis orienterede Jesper H. Andersen om den i MONIS 2 planlagte proces i for-hold til hvilke arter som evt. skal med på en dansk MSFD D2 Target Species List.

6.2 Fyto- og zooplankton: Diskussionen blev fokuseret på disse to organismegrupper. Efter en del snak frem og tilbage blev det tilkendegivet, at (1) der endnu ikke er mange erfaringer med eDNA-baseret overvågning af disse organismegrupper, og at (2) særlig opmærksomhed som udgangspunkt bør rettes mod følgende arter: (i) *Alexandrium* sp., (ii) *Pseudochattonella* sp. (iii) *Karenia* sp., (iv) 'dræbergoplen' (*Mnenopsis leydii*) og (v) kroghalet rovdafnie (*Cercopagis pengoi*).

6.3 Undervandsvegetation: Efter en kort diskussion blev det tilkendegivet, at (1) der er meget få erfaringer med eDNA-baseret overvågning af marin undervandsvegetation, og at (2) særlig opmærksomhed som udgangspunkt bør rettes mod følgende arter: (i) Gracilariatang (*Gracilaria sp.*) (ii) Saragassotang (*Saragassum muticum*), og (iii) rødalgen *Pophyra umbilicalis* sp).

6.4 Benthiske Invertebrateebrater m.v.: Efter en kort diskussion blev det tilkendegivet, at (1) der er nogle erfaringer med eDNA-baseret overvågning af marine Invertebrateebrater, bl.a. i relation til Vandrammedirektivet, og at (2) særlig opmærksomhed som udgangspunkt bør rettes mod følgende arter:

(i) Stillehavsøsters (*Crassostrea gigas*), (ii) svovlorm (*Marenzelleria* sp.), (iii) søpunge (*Didemnum* sp). og (iv) Kinesisk Uldhåndskrabbe (*Eriocheir sinensis*).

6.5 Fisk: Efter en del snak frem og tilbage blev det tilkendegivet, at (1) der allerede er en del positive erfaringer med eDNA-baseret overvågning af forskellige fisk, og at (2) særlig opmærksomhed som udgangspunkt bør rettes mod følgende arter: (i) sortmundet kutling (*Neogobius melanostomus*), (ii) pukkellaks (*Oncorhunchus gorbuscha*), og (iii) en række arter af stør.

6.6 Kriterier: Forslag til mulige kriterier for udabrjdelse af en dansk MSFD D2 Target Species List blev kort drøftet. Der var bred tilslutning til at listen skulle være transparent og kriteriebaseret.

7. Next steps – en åben diskussion

7.1 På baggrund at workshoppen vil der blive udarbejdet et kriterie-baseret udkast til en dansk MSFD D2 Target Species List. For så mange af arterne på denne liste som muligt vil der jf. referatets punkt 2.4 og 2.5 blive udviklet primers, som kan indgå i et Single Species Detection System.

7.2 Hvordan MONIS 2 skal afrapporteres er endnu ikke aftalt. NIVA Danmark vil derfor, i dialog med Naturstyrelsen, udarbejde et format herfor. I den forbindelse tilkendegav styrelsen, at afrapportering nok bør være på engelsk af hensyn til samarbejdet i regi af HELCOM og OSPAR.

8. Opsamling

8.1 NIVA Danmark vil udarbejde et referat og et udkast til en MSFD D2 Target Species List – begge vil blive sendt til samtlige deltagere i dagens workshop. Endelig takkede NIVA Danmark alle for aktiv deltagelse i workhoppen og ønskede god weekend ....

Appendix B: Draft Technical Guidance Report

1

Titel: Teknisk anvisning for indsamling af marine vandprøver og analyse for 'environmental DNA' (eDNA)			
Dokumenttype: Teknisk anvisning	TA. nr.: X01	Version: 1	Oprettet: 01.11.2015
Forfattere: - Steen W.Knudsen, Amphi Consult ApS/Afdeling for Evolutionary Genomics, Statens Naturhistoriske Museum, Københavns Universitet - Martin Hesselsøe, Amphi Consult Aps - Peter Rask Møller, Afdeling for Evolutionary Genomics, Statens Naturhistoriske Museum, Københavns Universitet - Jesper Andersen, NIVA Danmark	Gyldig fra: 01.05.2016 Sider: 16 Sidst ændret: 0x.0x.201x		
TA henvisninger	V07 – N12 – A02		

0 Indhold

2

1 Indledning

Denne tekniske anvisning er udarbejdet for indsamling af marine vandprøver med henblik på detektering af miljø-DNA ('environmental DNA' eller 'eDNA'), som beskrevet i flere aktuelle studier (f.eks. Ficetola et al., 2008; Goldberg et al., 2011; Olson et al., 2012; Thomsen et al., 2012a,b; Takahara et al., 2012; Pilliod et al., 2013; Taberlet et al., 2012, 2013; Deiner et al., 2015; Sigsgaard et al., 2015).

Den tekniske anvisning forudsætter for selve eDNA-analysen at der forudgående er designet artsspecifikke primere og prober – gennem *in silico* design (dvs. design gennem analyse af nukleotidsekvens data) – for den pågældende art, der ønskes detekteret og at eDNA i den indsamlede vandprøve forekommer i tilstrækkeligt høje koncentrationer til at kunne detekteres. Anvisningen er under stadig udvikling ved afdelingen for Evolutionary Genomics ved Statens Naturhistoriske Museum, Københavns Universitet, og bør derfor betragtes som foreløbig. Det skyldes bl.a. at det ikke er endeligt fastlagt uafklaret hvorledes eDNA bedst kan indsamles, opbevares og detekteres, da det i høj grad afhænger af både vandkvalitet, indsamling og opbevaring af vandprøve, ekstraktion af DNA og brugerens erfaring med både taxonomi og bioinformatik (jf. Deiner et al., 2015; Renshaw et al., 2015; Thomsen og Willerslev, 2015; Taberlet et al., 2012; Sigsgaard et al., 2015). Erfaringer ffra de først års overvågnign vil således medvirke til en løbende udvikling af anvisningen.

Denne anvisning bør som udgangspunkt kun følges såfremt indledende valideringstest (også kaldet *in vitro* test) er blevet udført for de benyttede primer- og probe systemer på DNA ekstraheret fra væv fra både den eftersøgte art, samt på DNA ekstraheret fra væv fra tætbeslægtede arter. Indledende *in vitro* test er påkrævet for ikke at risikere at fejlagtige konklusioner baseres på grundlag af falske positive signaler fra analysen.

2 Metode

Fremgangsmåden er delt i tre: (Del I) indsamling af vand, (Del II) ekstraktion af eDNA fra det indsamlede vand og (Del III) detektering af artsspecifik eDNA vha. kvantitative PCR (qPCR). Bemærk at mange studier benytter forskelige metoder for indsamling og ekstraktion af eDNA (jf. Ficotela et al., 2008; Takahara et al., 2012, 2013; Tréguier et al., 2014; Deiner et al., 2015). For at sikre at resultater er sammenlignelige på tværs af prøver, indsamling og arter, er det vigtigt at fremgangsmåden bliver standardiseret fra start.

Indsamling af vand (Del I) og ekstraktion af eDNA fra det indsamlede vand (Del II) kan som udgangspunkt gennemføres allerede nu. Detektering

artsspecifik eDNA vha. qPCR (Del III) forudsætter at både forudgående *in silico* design og grundig *in vitro* test er udført.

Del I. Indsamling af vand

Udbyttet af eDNA er umiddelbart koblet med mængden af indsamlet vand – jo mere vand, jo mere eDNA (Taberlet et al., 2012; Goldberg et al., 2015). Ved filtrering af vand anbefales det derfor, at så meget vand som muligt filtreres igennem en filterenhed. Alkoholfældning er et alternativ til filtrering – se bl. a. studier af Ficotela et al. (2008), Tréguier et al. (2014), Biggs et al. (2015), Deiner et al. (2015) for nærmere beskrivelse af denne fremgangsmåde. Eftersom både Amphi Consult og Statens Naturhistoriske Museum har bedst erfaring med filtrering, bygger denne anvisning udelukkende på filtrering, men det står brugeren frit for om alkoholfældning skal benyttes som et alternativ eller supplement til filtrering.

Den indsamlede filtrerede vandprøve skal p.t. opbevares i månedsvis eller årevis og skal derfor opbevares ved -80° C.

Filterenheden skal være steril inert og have en effektiv porestørrelse på 0.2–0.5 µm (se også Goldberg et al. 2011; Thomsen et al., 2012b). Filtret placeres i en integreret filterholder, som tillader læk-tæt samling med tryksat filtreringsenhed (f.eks. Amphiltrator, se www.amphi-consult.dk) ved et effektivt overtryk på minimum 1 bar atm (101 kPa), men gerne op imod 3 bar (304 kPa). Indsamles vandprøven fra en større beholder, er det nødvendigt at fjerne potentielt kontaminerende nukleinsyrer fra evt. tidligere vandprøvetagning. Nukleinsyrer (dvs. DNA) kan nedbrydes ved brug oxiderende reagenser ved vask med fortyndet brintoverilte eller fortyndet blegemiddel. Før vandprøven indsamles med filterenheden er det nødvendigt at fjerne overskydende oxiderende reagenser gennem gentagne vaske med havvand fra den pågældende prøveindsamlingslokalitet. Dette sikrer at filteret kun indsamler eDNA fra den pågældende indsamlingslokalitet. Amphi Consult er leveringsdygtig i udstyr der tillader tryksat filtrering med brug af engangsplastic der minimerer risikoen for kryds-kontamination uden brug af oxiderende reagenser eller andre kemikalier (se mere på www.amphi-consult.dk).

For at sikre validering af resultatet er det nødvendigt med flere replikater. Som minimum skal der indsamles tre filterprøve-replikater per lokalitet, men flere replikater kan med fordel indsamles. Vandindsamling, filtertype og præservering af filter skal standardiseres fra start for at sikre sammenlignelighed på tværs af både prøvetagningstidspunkt samt prøvetagningslokalitet.

4

Del II. Ekstraktion af eDNA fra det indsamlede vand
Ekstraktion af eDNA fra filtrerede vandprøver er baseret på DNeasy Blood & tissue kit (Qiagen), eftersom mange eDNA studier netop benytter dette ekstraktionskit (f.eks. Thomsen et al., 2012a,b; Takahara et al., 2013; Biggs et al., 2014; Tréguier et al., 2014; Sigsgaard et al., 2015). Amphi Consult og afdelingen for Evolutionary Genomics ved Københavns Universitet har sammenholdt DNeasy Blood & tissue kit (Qiagen) med NucleoSpin Soil (Macherey-Nagel). umiddelbart var DNeasy Blood & tissue kit (Qiagen) en anelse bedre til at oprense eDNA end NucleoSpin Soil (Macherey-Nagel) for marine vandprøver. Men dette resultat er stadig under analyse. Deiner et al. (2015) fandt også DNeasy Blood & tissue kit (Qiagen) gav bedst resultat.

Ekstraktionsprotokollen herunder kan også benyttes for alkoholfældede vandprøver (se også Ficotela et al. (2008) og Thomsen et al. (2012a) for detaljer.

Del III. In vivo detektering af eDNA ved kvantitativ PCR
Før kvantitativ detektion af eDNA kan indledes (*in vivo* test), er det strengt nødvendigt at grundige *in silico* og *in vitro* test er blevet udført og har sikret at primer- probe systemet ikke giver falske positive signaler pga. uønsket amplifikation af eDNA fra tætbeslægtede arter. Detaljeret *in silico* design kan ikke udelukke uspecifik uønsket amplifikation. Kun en detaljeret og grundig *in vitro* test kan sikre at fejlagtige konklusioner ikke drages på grundlag af falsk positiv amplifikation. Se også studierne af Taberlet et al. (2012), Wilcox et al. (2013) og Thomsen & Willerslev (2015).

Eftersom detektion af eDNA er baseret på et højt antal amplifikationscykler (>40 cykler) med 'annealing' og 'extension', kan primer og probe systemet detektere meget lave koncentrationer eDNA. Men ved >40 amplifikationscykler er der stor risiko for at få uønsket amplifikation fra tæt-beslægtede arter, der forekommer i samme habitat som den eftersøgte art (Wilcox et al., 2013). Vær opmærksom på at det ikke nødvendigvis er muligt at udlede fra et falsk positivt resultat fra qPCR, hvilken anden tætbeslægtet art, der har resulteret i uønsket amplifikation. Ønsket amplifikation kan desværre ikke skelnes fra uønsket amplifikation. Ydermere, skal brugeren være opmærksom på om *in silico* designet af primer- og probe systemet er baseret på ufuldstændig sammenholdning af nukleotid-sekvens data fra 'the National Center for Biotechnology Information (NCBI) GenBank. Arter der ikke var inkluderet i det indledende *in silico* design, kan heller ikke udelukkes som grundlag for mulig falsk positiv amplifikation, se også studiet af Wilcox et al. (2013).

En standard fortyndingsrække kan forberedes, hvis en kvantificering af eDNA i ekstraktionen fra filtervandprøven er ønsket (Ellison et al., 2006;

5

Natural History Museum of Denmark
University of Copenhagen

Amphi Consult

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Bustin et al., 2009; Turner et al., 2015). En standard fortyndingsrække kan tilberedes ved en indledende PCR på DNA ekstraheret fra den eftersøgte organisme og med de artsspecifikke primere der senere hen skal bruges for det efterfølgende qPCR setup. PCR-produktet kan derpå oprenses og koncentrationen på det oprensede PCR-produkt kan estimeres vha. spektrofotometri, Nanodrop, Qubit e.l. Eftersom nukleotid-sekvensen på PCR-produktet er kendt, kan molekylevægten beregnes, og antallet af PCR-produkter bestemmes som antal kopier per volumen. Dette oprensede PCR-produkt kan derpå fortyndes til en standard fortyndingsrække indledningsvis med 10^8 kopier per µL og i ti-fold fortyndinger (dvs. 10^7 kopier/uL, 10^6 kopier/uL osv. ned til 1 kopi/µL).

Den findes mange forskellige typer preproducerede mastermix for real-time PCR. Alle qPCR set ups udført ved afdelingen for Evolutionary Genomics ved Statens Naturhistoriske Museum over perioden 2012-2016 er baseret på TaqMan Environmental Master Mix 2.0 (Life Technologies), som også er foretrukket i mange studier på eDNA (Thomsen et al., 2012a,b; Tréguier et al., 2014; Sigsgaard et al., 2015; Turner et al., 2015). Derfor er denne protokol også baseret på TaqMan Environmental Master Mix 2.0 (Life Technologies).

Krydskontamination mellem forskellige qPCR kan forebygges ved brug af dUTP i alle qPCR produkter og addering af uracil DNA glycosylase (UDGase) til qPCR mix, med et indledende opvarmningstrin i qPCR programmet der inaktiverer UDGase (se Sigma Aldrich hjemmeside for yderligere detaljer om uracil DNA glycosylase). TaqMan Environmental Master Mix 2.0 (Life Technologies) indeholder ikke UDGase, og må derfor tilsættes separat. Tilsættes UDGase skal mængden af ddH_2O per reaktion justeres tilsvarende så total volumen forbliver 25µL per reaktion.

Reaktioner til qPCR forberedes i volumen mellem 25 µL til 100 µL. Her er 25 µL total volumen reaktioner anbefalet. Reaktionerne kan køres på en 'Stratagene Mx3005P quantitative PCR' maskine, eller på en tilsvarende 'real-time' qPCR maskine, der er i stand til at detektere de fluorescerende prober, der benyttes – dvs. maskinen skal kunne detektere FAM og ROX farve.

Skal forekomsten af eDNA vurderes med 95% konfidens-niveau, vil det være påkrævet at analysere minimum 19 positive qPCR-replikat-prøver ud af 20 qPCR-replikater-prøver per filtreret vandprøve, da 19/20*100% er lig med 95%. Dette kan hurtigt resultere i uforholdsvist mæssigt mange replikat-prøver, hvorfor en 'presence/absence' detektering uden mulighed for fastsættelse af konfidens-niveau umiddelbart anbefales. Se også studiet af Takahara et al. (2013) for flere detaljer om eDNA-detektering af akvatiske invasive arter. Ønskes blot 'presence/absence' detektering, skal

6

minimum fire qPCR-replikater analyseres per filter-vandprøve, men det anbefales kraftigt at analysere flere end blot fire qPCR-replikat-prøver per filtreret vandprøve.

2.1 Tid, sted og periode

For kystvande skal vandprøver indsamles fra midten af den opblandede vandsøjle, hvor fordelingen af eDNA antages som værende ensartet. Undgå vandindsamling nær bunden eller i sedimentet (Treguier et al., 2014; Turner et al., 2015). For lagdelte åbne farvande skal der indsamles vandprøver fra både over og under springlaget. For både kystvande og åbne farvande skal indsamlingen skal foregå på de stationer, der indgår i overvågningsprogrammet. Indsamlingerne skal ske to gange årligt, hhv. sent forår/tidlig sommer og sen sommer/tidlig efterår

2.2 Udstyr

Følgende udstyr skal bruges i forbindelse med prøvetagningen:

- Nukleinsyrefri vandhenter. F.eks. en spand først overskyllet med 5% hydrogen peroxide, og derpå overskyllet med 96% ethanol, og til sidst skyllet med vand fra den lokalitet, der skal samles ind fra.
- Sterile inerte filterenheder med effektiv porestørrelse på 0,2-0,5 µm
- Filterholder, som tillader læk-tæt samling med tryksat filtreringsenhed (f.eks. Amphiltrator - se www.amphi-consult.dk)
- Præserverende buffer f.eks. alkohol (96%), eller EDTA, eller LongMire Buffer (Longmire et al. 1997)
- Termometer. Skal rengøres og renses for nukleinsyrer ligesom vandhenter skal mellem hver prøvetagning.

Følgende udstyr skal anvendes til ekstraktionen:

- DNeasy Blood & tissue kit (Qiagen)
- ATL-buffer (komponent i Qiagen DNeasy Blood & tissue kit)
- Proteinkinase K (komponent i Qiagen DNeasy Blood & tissue kit)

Følgende udstyr skal anvendes til selve in vivo detektering af eDNA ved kvantitativ PCR

- Rotor eller mixer, der kan modstå opvarmning til mellem 50°-80° C i varmeskab.
- Varmeskab for inkubation ved temperaturer mellem 20°-80° C.
- Pipetter, i størrelser: P10, P20, P200 og P1000
- Sterile pipettespidser med filterbarriere. Spidserne skal matche størrelsesklasserne på pipetterne

7

- Spektrofotometer, Nanodrop, Qubit e.l. for mål af koncentration af DNA
- TaqMan Environmental Master Mix 2.0 (Life Technologies)
- dUTP (komponent i TaqMan Environmental Master Mix 2.0)
- Uracil DNA glycosylase (UDGasa)
- Stratagene Mx3005P quantitative PCR eller tilsvarende "real-time" qPCR-maskine, der kan detektere FAM og ROX-farve
- Bord-centrifuge, eller mini-centrifuge med maksimal hastighed på minimum 2000 rpm. Med plads til eppendorf-rør i 1,5-2,0 mL volumen
- Stor centrifuge med maksimal hastighed på minimum 14000 rpm. Med plads til eppendorf-rør i 1,5-2,0 mL volumen
- MX pro software for stratragene Mx3005P qPCR-maskine
- Plastrør for qPCR med plads til 100 uL reaktioner. Plastrør skal være i strips af otte.
- Klare plastlåg i strips af otte til plastrør. Plastlågen skal være flade, og tilpasset flourescens detektering i qPCR.
- Dobbelt distelleret RNase og DNase frit vand
- Sterile, RNase og DNase fri eppendorf rør i volumen 1,5-2,0 mL
- Primere og prober som angivet for de pågældende systemer

2.3 Procedure

I de følgende tre afsnit gennemgås proceduren for indsamling og filtrering af vand, ekstraktion af eDNA fra filtreret vandprøve, samt detektering af eDNA via en qPCR maskine og bearbejdning og analyse af detekteret eDNA data. Bemærk at analyse af data forudsætter kendskab til både metoder i kvantitativ PCR, samt begrænsninger for detektering af eDNA som blandt andet er beskrevet af Wilcox et al. (2013).

Fremgangsmåde for indsamling af vand:

Vand indhentes fra midten af vandsøjlen vha. en nukleinsyrefri vandhenter eller en nukleinsyrefri spand. En pumpe kan også bruges, men skal i såfald kunne renses for rester af nukleinsyrer fra tidligere indsamlingslokaliter så krydskontamination mellem indsamlingslokaliteter udelukkes. Amphi Consult kan vejlede i indsamling af vand, og tiltag for minimering af krydskontamination (se mere på www.amphi-consult.dk).

Indsamlet vand filtreres derpå gennem en steril nukleinsyrefri filtreringsenhed.

1) Benyt et sterilt filter med en effektiv porestørrelse på 0,2-0,5 µm som tillader filtration ved overtryk og stort filtreringsvolumen. Filtrér mellem 0,5 L og 1,5 L (Thomsen et al., 2012b). Mere end 1,5 L vand kan også filtreres så længe filteret ikke tilstoppes. Følg instruktion-

8

erne for den pågældende filterenhed. Man kan med fordel benytte udstyr, der integrerer både overtryk og filterenhed for at optimere filtrering i felten og laboratoriet (f.eks. en 'Amphiltrator', se mere på www.amphi-consult.dk).

2) Vandprøven indsamles fra midten af vandsøjlen, hvor fordelingen af eDNA antages som værende ensartet. Undgå vandindsamling nær bunden eller i sedimentet (Treguier et al., 2014; Turner et al., 2015).
3) Notér det filtrerede vandvolumen. Notér temperatur på vandet, samt dato for indsamling, og hvem der har indsamlet vandprøven.
4) Tøm filterenheden for evt. rest af vandprøve, så filterenheden er så tør som mulig inden filterenheden opbevares på køl og/eller med præserveringsbuffer (se Renshaw et al., 2015 for anbefalinger af typer af præserveringsbuffer).
5) Mærk filterprøven med unik nummerkode, for senere korrekt identifikation. Notér dato, indsamlingslokalitet, type af præseverende buffer der blev tilført filteret, opbevaringstemperatur og hvem der har indsamlet prøven.
6) Hold filterenheden kold (helst under -15°C, og som minimum under 0°C), indtil ekstraktion af DNA kan påbegyndes.
7) Overvej indsamling af flere replikater.

Fremgangsmåde for ekstraktion fra filtrerede vandprøver ved brug af DNeasy Blood & tissue kit (Qiagen):

8) For filtre opbevaret i buffer, må filteret først fjernes fra buffervæsken. Filteret skal udtørres under sterile forhold, evt. i et 'negative flow-hood' i 1-2 timer. Ubehandlede filtrer kan bruges direkte i næste trin.
9) ATL buffer og proteinase K tilsættes et rør med det udtørrede filter, som anbefalet i protokollen for DNeasy Blood & tissue kit (Qiagen). Mængden af ATL og proteinase K kan evt. justeres jf. med Thomsen et al. (2012b). Filter med ATL og proteinase K inkuberes på rotor eller mixer i varmeskab ved 56°C for minimum 2 timer så filtratet lyseres fuldstændigt.
10) Væsken med lyseret filtrat kan så overføres med pipette til 'spin columns' i DNeasy Blood & tissue kit (Qiagen), og oprenses ved at følge protokollen for DNeasy Blood & tissue kit (Qiagen).
11) For at sikre relativt høje koncentrationer af eDNA bør den sidste eluering ske i maksimalt 200 µL elueringsbuffer.
12) For langvarig opbevaring bør koncentrationen på det oprensede eDNA måles vha. spektrofotometri, Nanodrop, Qubit e.l.
13) Ekstraheret eDNA opbevares koldt (under -15° C) til alle tider, bortset fra når det skal bruges i qPCR set ups.

9

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Fremgangsmåde for detektering af artsspecifik eDNA vha. qPCR:

14) Som minimum skal fire replikater per prøve for hver ekstraheret filtreret vandprøve analyseres for en ' presence/absence' detektering uden mulighed for fastsættelse af konfidens-niveau. Bemærk at det kræver minimum 19 positive replikater ud af 20 replikater for at sikre 95% konfidens-niveau for tilstedeværelse af det eftersøgte eDNA.
15) Individuelle reaktioner klargøres til 25 µL total volumen, der består af 10 µL of TaqMan, Environmental Master Mix 2.0 (Life Technologies), 9 µL ddH2O, 3 µL ekstraheret DNA, 1 µL af hver primer (10 µM) og 1 µL probe (2.5 µM). Mere end 3µL ekstraheret DNA kan med fordel tilsættes for forøget chance for eDNA detektion, i så fald skal mængden af ddH2O nedjusteres tilsvarende per reaktion. F.eks. hvis der i stedet benyttes 5µL ekstraheret DNA, skal der kun tilføres 7 µL of ddH2O per reaktion. Som ved ordinær PCR set up, bør alle reagenser mixes og spinnes efter optøning (omkring 5-10 s ved 1000 rpm) i en lille bord-centrifuge, for at sikre at alle reagenser der tilføres er i de tiltænkte koncentrationer.
16) Minimum fire reaktioner skal tilsidesættes for 'non-target control' (NTC) test, for at sikre at alle reagenser er fri for kontamination.
17) 'Positive target control' (PTC) skal inkluderes i to til fire reaktioner. Inkluderes en standard fortyndningsrække kan den samtidig tjene som PTC. Ellers kan en PTC forberedes ved at tilsætte en fortynding af DNA ekstraheret fra væv fra den eftersøgte organisme.
18) En standard fortyndingsrække forberedes i ti-fold fortyndinger - dvs. i 107 kopier/µL, 106 kopier/µL, 105 kopier/µL, osv. ned til 100 kopier/µL. Hver ti-fold fortynding skal testes i minimum tre replikater. Jf. også med manualen til MxPro softwaren for Stratagene Mx3005P qPCR maskinen for fremstilling af standard fortyndingsrækker. Bemærk at en standard fortyndingsrække betragtes som unik per qPCR set up, og ikke kan overføres til lignende eller andre qPCR set ups. Se også anbefalinger fremført af Bustin et al. (2009) og Turner et al. (2015).
19) Programmet for qPCR skal være: 5 minutter ved 50° C, 10 minutter ved 95° C, og derpå 50 cykler med 95° C i 30 s og 60° C for 1 minut. Med 'end-point collection' af fluorescerens data under 60° C trinnet.
20) Da proberne er forsynet med BHQ1 og FAM-farve og TaqMan, Environmental Master Mix 2.0 (Life Technologies) indeholder ROX-farve sættes qPCR maskinen til at detektere FAM med ROX som reference.
21) Resultatet fra qPCR kørslen kvantificeres ved brug standard kurven udregnet fra standard fortyndingsrækken. Eftersom det oprindeligt

filtrerede vandvolumen er kendt, og volumen af tilsat elueringsbuffer er kendt, og volumen ekstraheret eDNA i den efterfølgende qPCR er kendt, kan mængden af eftersøgt eDNA i den oprindelige vandprøve beregnes, og i sidste ende benyttes som et vejledende mål for den relative forekomst af den eftersøgte organisme på den pågældende lokalitet. At benytte forekomsten af eDNA i den filtrerede vandprøve som mål for forekomsten af den eftersøgte organisme, vil kræve at der er indsamlet bestandsstørrelsesvurderinger for den eftersøgte art med konventionelle metoder samtidig med filtervandprøve er blevet indsamlet, og at disse samtidige indsamlinger af filtrer og bestandstørrelsesvurderinger er blevet gjort løbende igennem en længere årrække. Idet organismer må forventes at udskille eDNA med forskellig rate (se også Thomsen et al., 2012a,b), og få deres eDNA nedbrudt med forskellig rate med variation indenfor både arter, populationer, indsamlingstidspunkt og indsamlingslokalitet, er det ikke muligt at bestemme bestandsstørrelsen ud fra eDNA-mængden.

22) For kvantificering af kopier af det eftersøgte eDNA i den ekstraherede filtrerede vandprøve er det nødvendigt at skelne mellem 'limit of Detection' (LOD) og 'level of quantification' (LOQ). For at fastslå LOD og LOQ, må negative replikater i standard fortyndingsrækken først fjernes, da de ellers kan påvirke beregning af standardkurven. Dette kaldes også 'exclusion by sample' jf. studiet af Ellison et al. (2006). LOD er defineret som den laveste standard med positiv Ct-værdi (Cycle threshold) (se Bustin et al., 2009), hvor minimum én ud af flere qPCR replikater returnerer positive amplifikation - dette er som regel omkring den laveste koncentration af standard fortyndingsrækken, med kun én kopi per reaktion. Prøver med Ct-værdier over LOD bør betragtes som værende negative.
23) LOQ er defineret som laveste reproducerbare standard med positiv Ct-værdi, dvs. der hvor hver qPCR-replikat for den samme reaktion resulterer i positiv amplifikation - dette er som regel omkring 5-10 kopier per reaktion. Prøver med Ct-værdier over LOQ kan betragtes som værende positive (dvs. indenfor LOD), men kun mulige at kvantificere som mindre end den mindste reproducerbare standard (f.eks. < 5 eller 10 kopier per reaktion).
24) Efter LOD og LOQ er fastsat, kan mængden af eftersøgt eDNA per replikat i qPCR reaktionerne fra de ekstraherede filtrerede vandprøver kvantificeres forudsat Ct-værdierne er under LOQ.
25) Koncentration af eftersøgt eDNA i den oprindelige filtervandprøve kan derfor beregnes ved hjælp af følgende formel: Ae = Cqpcr * Fe * Vwf. Hvor Ae er antallet af eDNA-kopier per volumen filtreret vand, Cqpcr er koncentrationen af kopier af det eftersøgte eDNA i qPCR-reaktionen, Fe er brøkdelen af det eluerede ekstraherede filtrat benyttet i qPCR-reaktionen og Vwf er volumen af vand filtreret.

11

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2.4 Tjekliste

Ved vandindsamling:

- Vandhenter rengjort forinden
- Udstyr for vandindsamling klargjort
- Minimum 0,5 L vand er filtreret
- Notater omkring vandprøve er optegnet
- Filteret opbevaret i præserveringsbuffer, og hvilken buffer der i såfald er benyttet.
- Slut-filtrat er opbevaret ved < -15° C
- Hver filterprøve er mærket med unik nummerkode

Ved ekstraktion:

- Ekstraktionskit er klargjort forinden ekstraktion påbegyndes
- Yderligere remedier for ekstraktion er klargjort inden ekstraktionerne påbegyndes
- Notater for detaljer om ekstraktionerne er optegnet
- Hver ekstraktion er mærket med unik nummerkode
- Ekstraheret filterprøve er opbevaret ved <-15° C.

Ved qPCR analyse:

- Alle remedier og reagenser er klargjort
- Standardfortyndingrække er klargjort forinden
- Primere og prober er fortyndet til de korrekt 'working solutions' inden qPCR reaktionen sættes op
- Tilfredstillende in silico test og tilfredsstillende in vitro test er blevet gennemført inden test bliver forsøgt på filtrerede vandprøver. Således at der ikke er risiko for detektering af falske positive
- Notater er optegnet omkring indstillinger for qPCR opsætningen og for hvilke vandprøver der analyseres.
- qPCR analyse er kørt, og resulterende data-fil gemt under unikt filnavn.
- Ved 'presence/absence' studier er tilstedeværelse/fravær af den eftersøgt organisme noteret, og ved eDNA koncentrations-kvantificerings-forsøg er eDNA koncentrationer målt for hvert replikat i qPCR analysen for hver filterenhed.

2.5 Vedligehold af instrumenter

De fleste komponenter såsom plasticrør, Qiagen ekstraktionkit, filtrer, pipettespidser m.v. er engangsbrug. Benyttes en 'Amphiltrator' (www.amphi-consult.dk) for vandinsamling bør denne vedligeholdes som

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specificeret i manual og protokol. Pipetter bør vedligeholdes, kalibreres og renses i hht. til vejledninger for disse.

For vedligeholdelse af qPCR maskinen henvises også til manualen og softwaren for dette apparat.

Primere og prober, samt ekstraktioner fra filtrer og forberedte fortyndindingsrækker bør opbevares ved temperaturer under -15° C, for at sikre lang holdbarhed.

For brug og opbevaring af TaqMan EM Master Mix 2.0 henvises til vejledningen for dette produkt.

2.6 Særlige forholdsregler - faldgruber

For at kunne reproducere resultater og sammenholde mål for eDNA er det yderst vigtigt at både indsamling, opbevaring, ekstraktion og qPCR kvantificering fastsættes fra start. Afvigelser eller ændringer i protokollen kan influere mængden af eDNA kraftigt, og gøre sammenligninger umulige.

For sikker detektering er det påkrævet at have et stærkt kendskab til litteraturen omkring sporing vha. eDNA, og til taxonomien for den eftersøgte organisme såfremt *in silico* designet ikke er baseret alle tæt-beslægtede arter til den eftersøgte art. Derudover forudsættes det også at brugeren har en god forståelse for både det forudgående *in silico* design, og *in vitro* test, og et kendskab til hvordan en real-time PCR maskine fungerer og aflæses korrekt.

Falske positive signaler kan ikke umiddelbart skelnes fra sande positive. Der gives derfor ingen umiddelbar hurtig protokol for sikker eDNA-detektering af arter, men i stedet en række af vejledende retningslinjer der sammen med grundige overvejelser kan sikre korrekt detektering af eDNA.

3 Databehandling

Beskrivelse af dataflow herunder opmærksomhedspunkter.

3.1 Beregninger

Angivelse af hvordan beregninger med de indsamlede data skal foregå. Det drejer sig ikke om, de beregninger, hvor data skal indgå, men fx beregning af indeksværdi til breddevariationen og hvilken dybde blandingsprøverne til søkemi skal tages i.

13

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3.2 Data og koder

Liste over de data, der skal indlæses i database og angivelse af hvilken database.

For hvert filter skal følgende detaljer noteres. Forkortelseskoder for disse detaljer er angivet i parentes:

Indsamlingslokalitet, breddegrad og længdegrad (lok_pos):

- Filtertype benyttet (Fil_Typ)
- Temperatur på vandprøve inden filtrering (Temp_Inds)
- Dato for indsamling (Dato_inds)
- Navn på hvem der har indsamlet prøven (Navn_Inds)
- Dybdestrata for den indsamlede prøve (Dyb_Str)
- Volumen vand filtreret (Vwf)
- Unikt nummer for den pågældende prøve, for at sikre at replikat-prøver kan skelnes fra hinanden (U_Pr_Nr)
- Typen af præserveringsbuffer benyttet for at konservere filterprøven (Pr_Buff)
- Temperatur hvorunder filterprøven efterfølgende er opbevaret (Temp_F_Opb)

For hver ekstraktion af filtrer skal følgende noteres. Forkortelseskoder for disse detaljer er angivet i parentes:

- Unikt nummer for den pågældende prøve, skal være identisk med nummeret tildelt under filtrering (U_Pr_Nr)
- Dato for ekstraktion (Dato_eks)
- Ekstraktionskit benyttet (Eks_Kit)
- Volumen elueringsbuffer benyttet under ekstraktionen (Vol_EB)
- Temperatur hvorunder det ekstraherede filtrat opbevares (Temp_Ex_Opb)
- Målt koncentration på det ekstraherede eDNA (Cex_eDNA)

For hver kvantitativ PCR setup skal følgende data noteres. Forkortelses-koder for disse detaljer er angivet i parentes:

- Unikt fortløbende nummer tildelt pladen med alle qPCR reaktioner (Q_Nr)
- Filnavn tildelt pladen med qPCR reaktionerne. Et unikt nummer der også skal indeholde det unikke fortløbende nummer tildelt pladen qPCR-reaktionerne (QPl_No).
- Dato for qPCR (Dato_qpcr)
- Volumen på qPCR reaktioner (vol_qpcr_rxn)
- Eftersøgt art (Eft_art)

14

- Primer og probe system benyttet (prim_prob_sys)
- Antallet af reaktioner (Nr_rxn)
- Antallet af NTC reaktioner (NTC_rxn)
- Antallet af replikater for hver filtrat-ekstraktion (Repl_unk_rxn)
- Antallet af replikater for hver standard-fortyndingsrække (Repl_SD_rxn)
- Hvilke ekstraktioner af hvilke filtrater der er forsøgt analyseret i den pågældende qPCR, dvs. hvilke 'U_Pr_Nr' der er testet.
- Volumen af filtrat fra hver 'U_Pr_Nr'-esktraktion der er forsøgt testet per reaktionsrør (Vol_EksFil)
- Hvilken type real-time kvantitativ PCR maskine der er benyttet (Type_qpcr_mask)
- Hvem der har foretaget qPCR analysen (Resp_qPCR)

Eventuelt en liste med koder, der skal anvendes herunder hvilke standatkoder, der skal anvendes.

Hvis denne beskrivelse er den samme i mere end én TA, bliver dette beskrevet i en særskilt "databehanlings-TA". Afsnittet skal altid være i TA'en, men evt. kun med henvisninger til den "databehandlings-TA", der beskriver, hvordan data skal behandles.

4 Kvalitetssikring

4.1 Kvalitetssikring af metode

Beskrivelse af hvordan metoden kvalitetssikres.

4.2 Kvalitetssikring af data og dataaflevering

Beskrivelse af hvordan data kvalitetssikres.

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15

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16

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17

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6 Oversigt over versionsændringer

Version	Dato	Emne:	Ændring:
2.2	2011		Første version som anvendes til alle foregående TA'er
2.3	01.03.2012	Ændring	Ændret sidehoved og sidefod. DCE eller GEUS Logo i sidehovedet. Sidenummer i sidehoved.

Appendix C: *In silico* developed primers

The following pages describe species-specific primers / probe assays for each of the 50 species on the Target Species List.

The infomation presented includes:

1. Species number (following Table 2)

2. Species name

3. Species-specific primer/probe assay for the species in question with:

    a. target gene
    b. product size, melting temperature
    c. primer/probe length
    d. GC ratio (%)

4. Number of mismatches between primer and probe region in closely related non-target species

Species 1: Neogobius melanostomus

Full name: *Neogobius melanostomus* Pallas, 1814.

The genus *Neogobius* comprises four species and belongs to the family Gobiidae (Stepien & Nielson 2013; Froese & Pauly 2015).

An assay for *Neogobius melanostomus* has already been published and validated based on the mitochondrial cytochrome oxidase 1 (CO1) region and tested on digital droplet PCR (Nathan *et al.* 2014; Nathan *et al.* 2015). Annealing temperature used for this assay was 60 °C; the annealing temperatures in the table S1.1 below are the theoretical annealing temperature range calculated by Geneious version 9.0.4.

- Neo_Mel_CO1_F01: 5'-CTTCTRGCCTCCTCTGGWGTTG-3'
- Neo_Mel_CO1_R01: 5'-CCCWAGAATTGASGARATKCCGG-3'
- Neo_Mel_CO1_P01: 5'-FAM-FCAGGCAACTTRGCACATGCAG-BHQ-1-3'

Table S1.1: *Species-specific primer/probe assay for* Neogobius melanostomus *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Neogobius melanostomus	CO1	150 bp			
Neo_Mel_CO1_F01	CTTCTRGCCTCCTCTGGWGTTG		59.6 -62.8	22	54 -59
Neo_Mel_CO1_R01	CCCWAGAATTGASGARATKCCGG		58.9 -63.9	23	47-56
Neo_Mel_CO1_P01	CAGGCAACTTRGCACATGCAG		60.1 - 62.9	21	52 -57

Related species	**F01**	**R01**	**P01**
Neogobius melanostomus	0	0	0
Neogobius fluviatilis	1	1	0
Ponticola kessleri	2	0	1
Ponticola ratan	2	0	0
Ponticola rhodioni	2	1	2

Due to both the large degeneracy within the assays and better overall coverage on related species on the mitochondrial cytochrome b region a secondary assay was developed:

- Neo_Mel_Cytb_F01: 5'- TGCTTTAACCTCTCTCGCCC-3'
- Neo_Mel_Cytb _R01: 5'-GGAGTGACGAGGGGGTTTG-3'
- Neo_Mel_Cytb _P01: 5'-FAM-TCGGAGACCCAGACAACTTCATCCCAG-BHQ-1-3'

Beside the BLAST search of the assay components other native Gobidae species were also included in the alignments to improve and evaluate the assay, see table S1.2 on the next page.

Table S1.2: *Species-specific primer/probe assay for* Neogobius melanostomus *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Neogobius melanostomus	Cytb	84 bp			
Neo_Mel_Cytb_F01	TGCTTTAACCTCTCTCGCCC		59.8	20	55
Neo_Mel_Cytb_R01	GGAGTGACGAGGGGGTTTG		60.0	19	63
Neo_Mel_Cytb_P01	TCGGAGACCCAGACAACTTCATCCCAG		67.5	27	56

Related species	**F01**	**R01**	**P01**
Neogobius fluviatilis	3	1	4
Neogobius pallasi	4	2	3
Babka gymnotrachelus	4	3	3
Ponticola kessleri	6	4	3
Ponticola ratan	7	4	3
Ponticola platyrostris	9	4	5
Ponticola rhodioni	6	3	4
Gobius niger	7	3	4
Pomatoschistus minutus	8	4	6
	4	6	4

Species 2: Crassostrea gigas

Full name: *Crassostrea gigas* (Thunberg, 1793)

The genus *Crassostrea* comprises around 40 species worldwide (WoRMS Editorial Board 2015, Coan and Valentich-Scott 2012). The National Center for Biotechnology Information (NCBI) GenBank (Nov-2015) has sequences from 23 species of *Crassostrea* represented. The greatest diversity of *Crassostrea* on the NCBI GenBank database is found in sequence fragments of mitochondrial cytochrome oxidase 1 (mtDNA-CO1). The closest related species to *Crassostrea gigas* appears to be *Crassostrea angulata* found off Portugal (Batista *et al.* 2005) and *Crassostrea sikamea* off China (Wang *et al.* 2013). Also, *Crassostrea gigas* and *C. angulata* are known to hybridize (Batista *et al.* 2005).

Other mtDNA regions currently unavailable on NCBI, such as the mtDNA control region, might hold more variation and make it possible to distinguish between *C. gigas* and *C. angulate* in short stretches of mtDNA (80–110 bp long).
As the detection system recommended here is based on the mtDNA-CO1 fragment, this primer-probe system designed for *Crassostrea gigas* (Table S2) is likely to be unable to distinguish between *C. gigas* and *C. angulate* and *C. sikamea*, but should be able to distinguish *C. gigas*, *C. angulate,* C. *sikamea* from any other species of *Crassostrea*.

The following primers and probe are recommended as an assay for detecting eDNA from *Crassostrea gigas*, *C. angulate* and *C. sikamea*:

- Cra_gig_CO1_F05: 5'-AAGCCTTCACCTTGCTGGTA-3',
- Cra_gig_CO1_R05: 5'-CTAGTAAATGGCCCCCAACA-3',
- Cra_gig_CO1_P05: 5'-FAM-GCTCTATTTTCAGGTCAATTAATTTCA-BHQ-1-3'

Table S2: *Species specific primer/probe assay for* Crassostrea gigas *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Crassostrea gigas	CO1	95 bp			
Cra_gig_CO1_F05	AAGCCTTCACCTTGCTGGTA		59.9	20	50
Cra_gig_CO1_R05	CTAGTAAATGGCCCCCAACA		59.8	20	50
Cra_gig_CO1_P05	GCTCTATTTTCAGGTCAATTAATTTC		60.2	26	20

Related species	**Forward**	**Reverse**	**Probe**
Crassostrea angulata	1	0	1
Crassostrea ariakensis	5	6	2
Crassostrea belcheri	5	2	3
Crassostrea brasiliana	5	4	2
Crassostrea columbiensis	5	8	5
Crassostrea gasar	5	4	2
Crassostrea gigas	0	0	0
Crassostrea gryphoides	7	5	3
Crassostrea hongkongensis	4	2	3
Crassostrea iredalei	5	2	3
Crassostrea madrasensis	5	2	3
Crassostrea nippona	4	4	3
Crassostrea rhizophorae	7	3	3
Crassostrea sikamea	4	3	1
Crassostrea sikamea	4	3	1
Crassostrea virginica	6	4	3
Saccostrea cucullata	7	6	6

Species 3: Crepidula fornicate

Full name: *Crepidula fornicata* Linnaeus, 1758.

The genus *Crepidula* comprises 43 marine species and belongs to the family Calyptraeidae (WoRMS 2015). The CO1 region is the most suitable region for the assay as it has the best coverage of both target and non-target sister species, with CO1 sequence data available for 25 out of 43 species. The regions 16S, 18S and 28S were also assessed.

- Cre_For_CO1_F01: 5'- TCGGGGGATTTGGTAATTGGT -3'
- Cre_For_CO1_R01: 5'-CGGCCGAGGATAGCAATAGT -3'
- Cre_For_CO1_P01: 5'-FAM-AGGTGCTCCTGATATAGCTTTTCCTCGA-BHQ-1-3'

Based on the data available at NCBI the assay is specific, but specificity towards sister-species potentially occurring in Danish territorial waters cannot be excluded.

Table S3: *Species-specific primer/probe assay for* Crepidula fornicata *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	Gene	Product size	Temp	Length	GC
Crepidula fornicata	CO1	126			
Cre_For_CO1_F01	TCGGGGGATTTGGTAATTGGT		59.4	21	47.6
Cre_For_CO1_R01	CGGCCGAGGATAGCAATAGT		59.4	20	55
Cre_For_CO1_P01	AGGTGCTCCTGATATAGCTTTTCCTCGA		65.1	28	46.4

Related species	F01	R01	P01
Crepidula adunca	4	6	4
Crepidula arenata	5	6	4
Crepidula argentina	7	7	3
Crepidula onyx	8	8	8
Crepidula protea	7	7	3
Crepidula procellana	5	5	3
Crepidula complanata	4	5	4
Crepidula coquimbensis	4	6	2
Crepidula incurva	7	8	6
Crepidula marginalis	5	6	2
Crepidula philippiana	5	5	2
Crepidula cf. Perforans	5	6	2
Crepidula lessoni	4	6	2
Crepidula maculosa	4	8	5
Crepidula naticarum	4	8	1
Crepidula excavata	4	8	4
Crepidula atrasolea	6	8	3
Crepidula depressa	7	7	2
Crepidula plana	5	6	6
Crepidula cf. aplysioides	4	6	2
Crepidula navicula	5	6	2
Crepidula williamsi	3	5	3
Crepidula convexa	5	8	3
Crepidula unguiformis	5	7	5
Crepidula fimbriata	5	8	6

Species 4: Teredo navalis

Full name: *Teredo navalis* Linnaeus, 1758.

The genus *Teredo* comprises 15 marine species. The genus belongs to the family Teredinidae which contains three subfamilies and includes a total of 25 marine species (WoRMS 2015).

Presently, is *T. navalis* only represented by two sequences covering the 28S and 18S segment in the NCBI database. Only one (*T. clappi*) out of the 14 sister-species within the *Teredo* genus has available sequence data for the same regions. A comparison of the sequences reveals limited differentiation between *T. clappi* and *T. navalis* which potentially could lead to lack of separation between these two species. *Teredo clappi*, however, is only found in the western Atlantic, and will therefore not be a problem for specific detection of *T. navalis* in Dansih waters. Available data from several related species in other genera were also included in the alignments to improve the specificity of the assay. The following primers and probe are designed to target *T. navalis*:

- Ter_Nav_28S_F01: 5'-TGGATCCGCTACAACGATCG-3'
- Ter_Nav_28S_R01: 5'-CCGGAGAAAGTGCACCCTG-3'
- Ter_Nav_28S_P01: 5'-FAM-CGCACCGGGAGACGTCTCGTTCTCG-BHQ-1-3'

Table S4: *Species-specific primer/probe assay for* Teredo navalis *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Teredo navalis	28S	124			
Ter_Nav_28S_F01	TGGATCCGCTACAACGATCG		60.0	20	55
Ter_Nav_28S_R01	CCGGAGAAAGTGCACCCTG		60.7	19	63
Ter_Nav_28S_P01	CGCACCGGGAGACGTCTCGTTCTCG		70.6	25	68

Related species	**F01**	**R01**	**P01**
Teredo clappi	0	0	1
Dicyathifer manni	6	1	7
Neoteredo reynei	7	0	7
Teredothyra dominicensis	8	0	5
Lyrodus massa	2	0	1
Lyrodus pedicellatus	2	0	2

Species 5: Karenia mikimotoi

Full name: *Karenia mikimotoi* (Miyake & Kominami ex Oda) (G. Hansen & Ø. Moestrup 2000).

The genus *Karenia* comprises 9 species and an assay system has already been published for the target species within this genus (Yuan *et al.* 2012). The assay was designed for the Internal Trancribed Spacer 18S sequence:

- KMF: 5'-CTTTGTGTGTAACCTGTTGCTTTGT-3'
- KMR: 5'-TCAGCGGGTTTGCTTACCT-3'
- KMP: 5'-FAM-ACCTGTCCTCCTGTCTGCCACTTCATTTGT-BHQ-1-3'

The probe was initially designed as a TaqMan probe (Yuan *et al.* 2012), however, in this report all probes are reported as BHQ-1-probes.

The primers and probe that have been designed show strong species specificity to *Karenia mikimotoi* compared to the other eight "sister" species, as shown in table S5.

Table S5: *Species specific primer/probe assay for* Karenia mikimotoi *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Karenia mikimotoi	ITS	112 bp			
KMF	CTTTGTGTGTAACCTGTTGCTTTGT		62.5	25	40
KMR	TCAGCGGGTTTGCTTACCT		57.5	19	53
KMP	ACCTGTCCTCCTGTCTGCCACTTCATTTGT		72.1	30	50

Related species	**KMF**	**KMR**	**KMP**
K. asterichroma	18	11	18
K bidigitata	3+7 gasps	1	8
K. brevis	17	11	17
K. brevisulcata	19	11	17
K. cristata	17	11	14
K. papilionacea	16	11	16
K. selliformis	7 gaps	1	10
K. umbella	18	11	15

Species 6: Oncorhynchus mykiss

Full name: *Oncorhynchus mykiss* (Walbaum, 1792).

The genus *Oncorhynchus* belongs to the family Salmonidae and comprises 15 species (Froese and Pauly, 2015)

Occurrences of hybridization between *O. mykiss* and *Salmo salar* have been reported. Since the mitochondrial genome of these hybrids is maternally inherited the assay will be unable to distinguish hybrids from normal individuals in cases where the mitochondrion genome originates from *O. mykiss* (Wang *et al.* 2015). *Oncorhynchus mykiss* showed high similarity to *O. gilae* and *O. Chrysogaster* both with regard to the CO1 and Cytochrome b sequences. However, as the latter two species are of Pacific Ocean origin and neither occurs in Danish territorial waters, they do not pose a challenge for false positives. The following primers and probe are designed to target *O. mykiss:*

- Onc_Myk_Cytb_F01: 5'-ACCTCCAGCCATCTCTCAGT-3'
- Onc_Myk_Cytb_R01: 5'-AGGACGGGGAGGGAAAGTAA-3'
- Onc_Myk_Cytb_P01: 5'-FAM-TGAGCCGTGCTAGTTACTGCTGTCCTT-BHQ-1-3'

Table S6: *Species-specific primer/probe assay for* Oncorhynchus mykiss *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Oncorhynchus mykiss	Cytb	90 bp			
Onc_Myk_Cytb_F01	ACCTCCAGCCATCTCTCAGT		60.0	20	55
Onc_Myk_Cytb_R01	AGGACGGGGAGGGAAAGTAA		59.9	20	55
Onc_Myk_Cytb_P01	TGAGCCGTGCTAGTTACTGCTGTCCTT		67.6	27	52

Related species	**F01**	**R01**	**P01**
Oncorhynchus gorbuscha	3	3	4
Oncorhynchus mykiss x Salmo salar_KP218514	0	0	0
Oncorhynchus mykiss x Salmo salar_NC_026537	0	0	0
Onchorhynchus gilae	0	0	0
Oncorhynchus chrysogaster	0	0	0
Oncorhynchus keta	3	5	5
Oncorhynchus clarkii	3	1	2
Oncorhynchus nerka	2	3	4
Oncorhynchus tshawytscha	3	3	3
Oncorhynchus kisutch	3	4	3
Oncorhynchus masou	4	3	3
Salmo trutta	3	4	6
Salmo salar	2	3	5

Species 7: Mnemiopsis leidyi

Full name: *Mnemiopsis leidyi* Agassiz, 1865

The species *Mnemiopsis leidyi* is the only species within the genus *Mnemiopsis*. Within the nearest species under the Order *Lobata* the 18S sequence could not be used to differentiate between *Mnemiopsis leidyi* and non-target species. Therefore, to ensure sufficient specificity of the assay designed towards *Mnemiopsis leidyi*, a Blast search was conducted using several CO1 sequence available through the NCBI database. An eDNA primer/probe assay was designed based upon the alignment of in total 35 sequences from different non-target species. The assay showed a high degree of mismatches to non-target species (table S7):

- Mne_lei_CO1_F01: 5'-CTCATGTTTTGTCATTTTTAATTAAC-3'
- Mne_lei_CO1_R01: 5'-CTAAACAACCTAATAAACCTATAC-3'
- Mne_lei_CO1_P01:5'-FAM-AATGTACCTTTTTCTTATCCTGGCTTAAAT-BHQ-1-3'

Table S7: *Species specific primer/probe assay for* Mnemiopsis leidyi *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species*

Species	Gene	Product Size	Temp	Length	GC
Mnemiopsis leidyi	CO1	114 bp			
Mne_lei_CO1_F01	CTCATGTTTTGTCATTTTTAATTAAC		56.8	26	23
Mne_lei_CO1_R01	CTAAACAACCTAATAAACCTATAC		56.6	24	29
Mne_lei_CO1_P01	AATGTACCTTTTTCTTATCCTGGCTTAAAT		63.9	30	30

Related species*	Forward	Reverse	Probe
Cotesia salebrosa	15	9	14
Cotesia salebrosa voucher Cs_III_3	15	9	14
Cotesia salebrosa voucher Cs_II_1	15	9	14
Cotesia salebrosa voucher Cs_IV_1	15	9	14
Cotesia salebrosa voucher Cs_VII_8	15	9	14
Cotesia marginiventris	15	9	15
Cotesia griffini	15	8	15
Cotesia melitaearum	15	7	14
Aloeides arida	15	8	14
Ampedus ainu	15	-	15
Aphonopelma mojave	13	10	15
Callicore astarte	15	9	14
Catoblepia amphirhoe	15	8	13
Conogethes punctiferalis	14	9	15
Baryscapus servadeii	14	8	16
Hiatella sp. L HML-2015	13	10	17
Idarnes sp. ex Ficus goldmanii	17	5	13
Ischyropsalis adamii	15	9	10
Limenitis helmanni	16	11	12
Limoniscus atricolor	16	-	10
Lanxoblatta emarginata	15	6	13
Mazocraeoides gonialosae	13	7	17
Osmia juxta	14	8	12
Phenacoccus baccharidis	17	10	18
Principapillatus hitoyensis	15	9	16
Pauropsalta infuscata	16	10	15
Pterolophia rigida	15	9	15
Panorpodes paradoxus	14	8	15

Psepholax leoninus	16	9	16
Psepholax mastersii	16	9	16
Spermophagus brevipes	16	9	14
Spermophagus cornutus	15	10	16
Torymus beneficus	14	9	14
Telenomus rowani	15	-	6
Yramea lathonoides	15	9	16

* not necessary related but show strong sequence similarity to target species

Species 8: Eriocheir sinensis

Full name: *Eriocheir sinensis* Milne-Edwards, 1853

According to WoRMS the genus *Eriocheir* comprises four marine species. The genus belongs to the family Varunidae which contains six subfamilies and 36 genera (WoRMS 2015).

Sequence information is available for both *E. sinensis* and its sister-species. Beside a BLAST search of the assay, other native decapod species were included in the evaluation of the assay, see Table S8. Due to limited variation between *E. sinensis* and *E. Japonica* the assay cannot separate these two species. However, both *E. Japonica* and *E. ogasawaraensis* only occur in the western Pacific (Yamasaki *et al.* 2006; Komai *et al.* 2006; WoRMS 2015) and are therefore not expected to result in false positives for samples collected in Danish territorial waters. The following primers and probe are designed to target *E. sinensis:*

- Eri_Sin_Cytb_F01: 5'-TCGGTACCGACCTAGTACAA-3'
- Eri_Sin_Cytb_R01: 5'-AGAAAATGCTGATGCTACTAAAGGT-3'
- Eri_Sin_Cytb_P01: 5'-FAM-TGAGGAGGGTTTTCTGTTGATAATGCCAC-BHQ-1-3'

Table S8: *Species-specific primer/probe assay for* Eriocheir sinensis *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Heterosigma akashiwo	Cytb	113 bp			
Eri_Sin_Cytb_F01	TCGGTACCGACCTAGTACAA		57.2	20	50
Eri_Sin_Cytb_R01	AGAAAATGCTGATGCTACTAAAGGT		58.8	25	36
Eri_Sin_Cytb_P01	TGAGGAGGGTTTTCTGTTGATAATGCCAC		65.3	29	45

Related species	**F01**	**R01**	**P01**
Eriocheir ogasawaraensis	1	0	3
Eriocheir japonica	0	0	0
Eriocheir hepuensis	2	0	1
Paralithodes camtschaticus	8	10	6
Nephrops norvegicus	5	11	5
Homarus gammarus	5	10	5
Homarus americanus	5	9	5

Species 9: Rhithropanopeus harrisii

Full name: *Rhithropanopeus harrisii* (Gould, 1841)

The north American mud crab (*R. harrisii*) is a monotypic species for Rhithropanopeus (Türkay 2001, WoRMS Editorial Board 2015). The family Panopeidae holds 27 genera, and 22 of these are represented by various sequences on the NCBI database. A comparative study on mitochondrial haplotypes found in R. *harrisii* in Eastern Denmark (Aagaard 2015) and a quick comparison of cytochrome oxidase 1 (CO1) sequences from NCBI infers a close relationship between *Rhithropanopeus*, *Eurypanopeus* and *Hexapanopeus*.

For the CO1 gene in the mt-genome, there appear to be five haplotypes of *R. harrisii* present in Danish seas (Aagaard 2015). The primer-probe system recommended here is assumed to be able to differentiate between *Rhithropanopeus*, *Eurypanopeus* and *Hexapanopeus*. A European checklist (Türkay 2001) lists only *Dyspanopeus sayi* (Smith, 1869), *Panopeus africanus* A. Milne-Edwards, 1867, and *Rhithropanopeus harrisii* from the family Panopeidae in European coastal seas. This limits the number of potential non-target species that might be co-occurring with the target-species (*R. harrisii*), and thus reduces the risk of obtaining false positives from other representatives of Panopeidae. The primers and probes recommended here are matched against CO1 sequences from multiple representatives of Panopeidae (Table S9).

- Rhi_har_CO1_F01: 5'-CCACCATCCCTTACACTCCT-3',
- Rhi_har_CO1_R01: 5'-CTCCTGCATGAGCAATAGCA-3',
- Rhi_har_CO1_P01: 5'-FAM-GAAAGAGGAGTTGGAACAGGATGAACTG–BHQ-1-3',

Table S9: *Species specific primer/probe assay for* Rhithropanopeus harrisii *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Rhithropanopeus harrisii	CO1	106 bp			
Rhi_har_CO1_F01	CCACCATCCCTTACACTCCT		58.9	20	55
Rhi_har_CO1_R01	CTCCTGCATGAGCAATAGCA		60.1	20	50
Rhi_har_CO1_P01	GAAAGAGGAGTTGGAACAGGATGAACTG		67.2	28	46

Related species	**Forward**	**Reverse**	**Probe**
Acantholobulus bermudensis	6	1	0
Acantholobulus pacificus	3	2	1
Acantholobulus schmitti	2	2	1
Chasmophora macrophthalma	4	1	4
Cyrtoplax panamensis	4	6	4
Dyspanopeus sayi	2	4	1
Dyspanopeus texanus	2	3	1
Eurypanopeus abbreviatus	4	4	1
Eurypanopeus depressus	2	2	1
Eurypanopeus dissimilis	2	4	2
Eurypanopeus ovatus	3	4	1
Eurypanopeus planissimus	4	6	2
Eurypanopeus planus	4	4	4
Eurypanopeus turgidus	2	2	1
Eurytium affine	3	5	2
Eurytium albidigitum	3	4	2
Eurytium tristani	2	5	1
Glyptoplax pugnax	2	2	2
Glyptoplax smithii	3	3	2
Hexapanopeus angustifrons	3	4	3

Hexapanopeus caribbaeus	2	3	2
Hexapanopeus lobipes	5	2	5
Hexapanopeus paulensis	2	3	4
Lophopanopeus bellus	2	2	4
Lophoxanthus lamellipes	4	4	6
Malacoplax californiensis	5	3	1
Metopocarcinus concavatus	3	3	2
Neopanope packardii	2	5	3
Panopeus africanus	2	4	1
Panopeus americanus	2	3	1
Panopeus austrobesus	3	5	1
Panopeus chilensis	2	4	1
Panopeus hartii	2	3	1
Panopeus herbstii	2	4	1
Panopeus lacustris	3	5	1
Panopeus obesus	2	5	1
Panopeus occidentalis	2	4	1
Panopeus purpureus	3	5	1
Panopeus rugosus	3	5	1
Panopeus simpsoni	2	3	1
Panoplax depressa	3	4	1
Rhithropanopeus harrisii	0	0	0
Robertsella mystica	5	2	5
Tetraplax quadridentata	3	4	2
Tetraxanthus rathbunae	1	4	1
Thalassoplax angusta	5	2	5

Species 10: Prorocentrum minimum

Full name: *Prorocentrum minimum* (Pavillard) Schiller, 1933

Prorocentrum minimum belongs to the genus *Prorocentrum* that comprises 85 species. Primer/probe assay was designed after Scorzetti *et al.* (2009). The authors had selected the D1D2 region of the 28S sequence, which commonly selected for assay design for algae species (e.g., Dittami *et al.* 2013). The only modification to the otherwise good assay was the design of a new reverse primer to potentially provide better species-specificity to *Prorocentrum minimum*. In addition three extra bases were added to the probe to increase temperature and make it longer than the two primers:

- Pro_min_28S_F01: 5'-GGGTCATGGTAGCTCGTCTA-3'
- Pro_min_28S_R01: 5'-CAGGTCCCCCGCCAGT-3'
- Pro_min_28S_P01 (PI3*): 5'-GCAGTGTCCTTGGCATTCTGGAAG-3'
  * original probe name without AAG at the end (Scorzetti *et al.* 2009)

Besides the perfect sequence similarity to *P. balticum* the number of base pair mismatch to closest related species, where it was possible to align sequences, was high (Table S10). In total 34 species within the genus *Prorocentrum* had been sequenced for the gene region of interest in the assay design. Please note that for the probe the number of mismatches to non-target sister species was not recorded due to 11 extra bases in several of these non-target species. However, mismatch in the direct comparable sequence was high (6–12 bp) for all, except *P. balticum* (0 bp).

Table S10: *Species specific primer/probe assay for* Prorocentrum minimum *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species (- = missing nucleotide possibly due to gap insertion for alignment in BioEdit (Hall 1999)). ND = not done due to large gap insertion. Problematic species are marked in bold*

Species	Gene	Size	Temp	Length	GC
Prorocentrum minimum	28S	134 bp			
Pro_min_28S_F01	GGGTCATGGTAGCTCGTCTA		60.5	20	55
Pro_min_28S_R01	CAGGTCCCCCGCCAGT		58.4	16	75
Pro_min_28S_P01	GCAGTGTCCTTGGCATTCTGGAAG		66.9	24	54

Related species	Forward	Reverse	Probe
Prorocentrum arabianum	11	9	ND
Prorocentrum arenarium	10	11	ND
Prorocentrum balticum	**0**	**0**	**ND**
Prorocentrum bimaculatum	10	12	ND
Prorocentrum concavum	11	9	ND
Prorocentrum consutum	9	12	ND
Prorocentrum dentatum	2	5	ND
Prorocentrum donghaiense	2	5	ND
Prorocentrum elegans	4	-	ND
Prorocentrum emarginatum	11	-	ND
Prorocentrum emarginatum/fukuyoi	10	8	ND
Prorocentrum faustiae	11	9	ND
Prorocentrum foveolatum	6	14	ND
Prorocentrum fukuyoi	11	10	ND
Prorocentrum gracile	4	-	ND
Prorocentrum hoffmannianum	11	11	ND
Prorocentrum levis	9	13	ND
Prorocentrum lima	11	11	ND

Prorocentrum mexicanum	4	8	ND
Prorocentrum micans	7	11	ND
Prorocentrum panamense	9	13	ND
Prorocentrum playfairi	6	-	ND
Prorocentrum rhathymum	4	8	ND
Prorocentrum sculptile	4	8	ND
Prorocentrum sigmoides	4	12	ND
Prorocentrum sp. CCMP1724	11	9	ND
Prorocentrum sp. IRTA002	6	13	ND
Prorocentrum sp. SM19	9	10	ND
Prorocentrum sp. Y8	11	9	ND
Prorocentrum texanum	6	11	ND
Prorocentrum triestinum	7	11	ND
Prorocentrum compressum	6	9	ND

Species 11: Gracilaria vermiculophylla

Full name: *Gracilaria vermiculophylla* (Ohmi) Papenfuss (1967)

The genus *Garcilaria* contains 99 species and variates. The most available sequence from these species was the *rbc*L gene. For some species this was the only sequences gene. However, for the following *Garcilaria* species the rbcL gene was not available: *G. abbottiana, G. brasiliensis, G. cearensis, G. confervoides, G. crassa, G. dawsonii, G. dentata, G. epihippisora, G. ferox, G. foliifera var. angustissima, G. aff. lacinulata, G. aff. mammillaris, G. manilaensis,* and *G. aff. tepocensis.*

Sequence information from the following species could not be aligned:
G. cliftonii, G. corticata, G. corticata var. corticata, G. delibis, G. denticulate, G. eucheumoides, G. fergusonii, G. foliifera, G. gigas, G. millardentii, and *G. robusta.*

The following species showed identical sequences to *Gracilaria vermiculophylla* :
G. asiatica (AY769260.1, AY769261.1), *G. tenuistipita* (AY049312.1) and *G. tenuistipitata*, (AY049313.1, AY049314.1, AY049324.1). Some confusion remains around *G. textorii* since HQ880647.1 shows on differentiation to target sequence, whereas DQ095814.1 (shown in table S11.1) shows decent differentiation.

Based on the sequences available in NCBI and that could be aligned the following assay was designed:

- Gra_ver_rbcL_F01: 5'-CTTTCCAGGGGCCAGCA-3'
- Gra_ver_rbcL_R01: 5'-AAAGGACGACCAAACTTATCC-3'
- Gra_ver_rbcL_P01: 5'-FAM-GGACTGGTTGTAGAACGTGAACGTAT-BHQ-1-3'

The assays base pair differentiation to related species was 3.7, 2.1, and 4.0 on average for the F01, R01, and P01 primers and probe, respectively. The exact differences are listed in table S11.1. Please note here that the assay could not be designed to not target *Gracilaria asiatica.*

Table S11.1: *Species specific primer/probe assay for* Gracilaria vermiculophylla *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species. Problematic species are marked in bold*

Species	Gene	Product size	Temp	Length	GC
Gracilaria vermiculophylla	rbcL	67 bp			
Gra_ver_ rbcL_F01	CTTTCCAGGGGCCAGCA		57.3	17	65
Gra_ver_ rbcL_R01	AAAGGACGACCAAACTTATCC		57.5	21	43
Gra_ver_ rbcL_P01	GGACTGGTTGTAGAACGTGAACGTAT		66.2	26	46

Related species	F01	R01	P01
Gracilaria apiculata	4	2	4
Gracilaria arcuata	3	1	5
Gracilaria armata	4	3	4
Gracilaria asiatica	**0**	**0**	**0**
Gracilaria babae	3	1	4
Gracilaria beckeri	3	0	7
Gracilaria birdiae	4	3	3
Gracilaria blodgettii	6	2	3
Gracilaria bursa-pastoris	3	3	5
Gracilaria cacalia	3	1	4
Gracilaria canaliculata	3	1	4
Gracilaria capensis	3	1	5
Gracilaria caudata	4	2	2

Gracilaria cervicornis	4	2	4
Gracilaria changii	6	2	2
Gracilaria chilensis	4	2	4
Gracilaria chouae	4	3	5
Gracilaria conferta	6	3	4
Gracilaria cornea	4	3	3
Gracilaria coronopifolia	4	4	5
Gracilaria crassissima	4	3	3
Gracilaria cuneata	4	2	3
Gracilaria cuneifolia	4	2	5
Gracilaria curtissiae	4	2	3
Gracilaria cylindrica	4	1	4
Gracilaria damaecornis	4	3	4
Gracilaria domingensis	4	2	4
Gracilaria dotyi	3	4	5
Gracilaria dura	4	2	3
Gracilaria edulis	4	2	4
Gracilaria eucheumatoides	4	1	3
Gracilaria firma	6	2	3
Gracilaria flabelliforme	4	3	4
Gracilaria gracilis	4	3	3
Gracilaria galetensis	4	3	5
Gracilaria hayi	4	2	5
Gracilaria huangii	3	1	4
Gracilaria incurvata	3	2	5
Gracilaria intermedia	3	1	5
Gracilaria irregularis	5	2	2
Gracilaria isabellana	4	1	4
Gracilaria lacinulata	4	1	5
Gracilaria longa	3	2	4
Gracilaria mammillaris	4	4	5
Gracilaria multipartita	2	2	4
Gracilaria occidentalis	4	3	4
Gracilaria oliveirarum	4	3	5
Gracilaria ornata	4	3	4
Gracilaria pacifica	4	2	4
Gracilaria parvispora	4	3	5
Gracilaria punctata	4	2	3
Gracilaria rhodymenioides	4	2	5
Gracilaria salicornia	3	1	4
Gracilaria secundata	4	2	4
Gracilaria smithsoniensis	4	3	5
Gracilaria spinulosa	6	1	5
Gracilaria stipitata	3	1	5
Gracilaria taiwanensis	4	3	4
Gracilaria tenuistipitata	4	1	4
Gracilaria textorii	3	2	4
Gracilaria tikvahiae	4	2	5
Gracilaria truncata	4	2	4
Gracilaria urvilleii	4	1	2
Gracilaria usneoides	4	3	3
Gracilaria venezuelensis	3	1	5
Gracilaria viellardii	4	3	4
Gracilaria yoneshigueana	3	2	5

Due to relatively large proportions of missing data for *rbc*L gene, an attempt to construct an assay for CO1 was also undertaken. However, a smaller proportion of the species have been sequenced for CO1 compared to *rbc*L (table S11.1 vs. table S11.2). The assay for *Gracilaria vermiculophylla* based on CO1:

- Gra_ver_CO1_F02: 5'-CGTCAGCAGTTGTAGAAGTC-3'
- Gra_ver_CO1_R02: 5'-AGAGGCTCCTGATATGTGC-3'
- Gra_ver_CO1_P02: 5'-FAM-CTCAGGCGGAGCTGTAGATTTAGCT-BHQ-1-3'

Despite the lower number of sequences species within the genus *Gracilaria* the designed assay showed on average higher degrees of differentiation to sister species compared to *rbc*L (please note that the list of sister species is not directly comparable between tables S11.1 and S11.2). Averages were F02 2.9, R02 4.5, and P02 6.4. However, *Gracilaria corticata* and *Gracilaria foliifera* showed none or only one mismatch to the designed assay. In general these two species showed very little mismatch in the sequence and not in positions that would make the design of an assay possible.

Table S11.2: *Species specific primer/probe assay for* Gracilaria vermiculophylla *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species. Problematic species are marked in bold*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Gracilaria vermiculophylla	CO1	109 bp			
Gra_ver_CO1_F02	CGTCAGCAGTTGTAGAAGTC		58.4	20	50
Gra_ver_CO1_R02	AGAGGCTCCTGATATGTGC		57.5	19	53
Gra_ver_CO1_P02	CTCAGGCGGAGCTGTAGATTTAGCT		67.4	25	67.4

Related species	**F02**	**R02**	**P02**
Gracilaria corticata	**1**	**0**	**0**
Gracilaria foliifera	**0**	**0**	**0**
Gracilaria flabelliformis subsp. simplex	4	4	5
Gracilaria arcuata	2	5	8
Gracilaria manilaensis	4	4	8
Gracilaria salicornia	1	6	5
Gracilaria smithsoniensis	5	4	5
Gracilaria hayi	4	6	6
Gracilaria dentata	4	3	7
Gracilaria damaecornis	4	4	7
Gracilaria curtissiae	4	6	6
Gracilaria cervicornis	5	3	8
Gracilaria birdiae	4	5	5
Gracilaria gracilis	3	4	7
Gracilaria chouae	6	5	4
Gracilaria textorii	5	4	8
Gracilaria cornea	5	5	5
Gracilaria yoneshigueana	4	5	7
Gracilaria isabellana	4	4	6
Gracilaria ferox	5	3	7
Gracilaria domingensis	5	3	8
Gracilaria cuneata	4	5	8
Gracilaria caudata	5	3	7
Gracilaria pacifica	4	4	8
Gracilaria tenuistipitata var. liui	4	6	6
Gracilaria chouae	6	5	4
Gracilaria blodgettii	6	4	6
Gracilaria salicornia	2	6	5

Species 12: Sargassum muticum

Full name: *Sargassum muticum* (Yendo) Fensholt, 1955

Within the Genus *Sargassum* there are 121 species and variates. Due to the large number of species within this genus it was very challenging to identify a gene with enough variation between *Sargassum muticum* and the remaining members. Despite large availability of genes within this genus it was not possible to design any assay using sequence information from the following genes *rbc*L, 28S, *psa*A, ITS, and CO1. After extensive search the gene region COX3 was identified as a good alternative for designing an assay.

Several possibilities to position elements of an assay were identified within the published sequences for COX3 showing a good amount of mismatches to non-target speices. However, the majority of these showed the potential for secondary structures or self-annealing, and could therefore not with confidence be used to construct an assay. Despite this it was possible to design an assay for *Sargassum muticum* from the COX3 gene, however with relatively modest numbers of mismatch to non-target species, as represented in table S12.

- Sar_mut_COX3_F01: 5'-TGCACCTTTCGCTATGTCC-3'
- Sar_mut_COX3_R01: 5'-GGTACCAATAATAACATGGAAAC-3'
- Sar_mut_COX3_P01: 5'-FAM-CAGTGTTTATGGTTCTGTTTTTTTTATGGC-BHQ-1-3'

In the event that the assay should not be specific enough to *Sargassum muticum* an alternative primer pair without probe was designed. A probe could not be design due to formation of secondary structures preventing annealing to target strand. Despite the lack of a probe the primer pair should be successful due to a higher degree of mismatch to non-target species (not shown) compared to assay system 1 (above):

- Sar_mut_COX3_F02: 5'-TTATTTTACTTTCTTCCGGCGCT-3'
  57.6°C, 25 bp long, and GC% = 28.
- Sar_mut_COX3_R02: 5'-CAAATATTATTGTAATGAGCAAACTT-3'
  56.8°C, 26 bp long, and GC% = 23.
  Product length including primers, 104 bp

Table S12: *Species specific primer/probe assay for* Sargassum muticum *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species. Problematic species are marked in bold*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Sargassum muticum	COX3	84 bp			
Sar_mut_COX3_F01	TGCACCTTTCGCTATGTCC		57.5	19	53
Sar_mut_COX3_R01	GGTACCAATAATAACATGGAAAC		57.6	23	35
Sar_mut_COX3_P01	CAGTGTTTATGGTTCTGTTTTTTTTATGGC		65.1	30	33

Related species	**F02**	**R02**	**P02**
Sargassum aquifolium	4	6	2
Sargassum autumnale	3	6	4
Sargassum binderi	4	6	2
Sargassum brandegeei	3	5	3
Sargassum carpophyllum	3	5	2
Sargassum cf. cymosum	3	5	2
Sargassum cf. latifolium	3	6	2
Sargassum cf. polyceratium	3	5	2
Sargassum cf. yamamotoi	4	5	4
Sargassum coreanum	4	5	4
Sargassum cymosum	3	5	2

Sargassum cymosum var. scabriusculum	3	5	2
Sargassum echinocarpum	4	6	2
Sargassum elegans	3	5	2
Sargassum fallax	3	6	4
Sargassum filicinum	1	3	1
Sargassum filipendula	3	5	2
Sargassum fluitans	3	5	2
Sargassum fulvellum	**1**	**1**	**1**
Sargassum fusiforme	4	6	2
Sargassum giganteum	3	5	2
Sargassum hemiphyllum	2	5	1
Sargassum herporhizum	3	5	3
Sargassum horneri	1	3	1
Sargassum horridum	3	5	3
Sargassum howeanum	3	5	2
Sargassum hystrix	3	5	2
Sargassum ilicifolium	3	5	2
Sargassum ilicifolium var. acaraeocarpum	3	5	1
Sargassum johnstonii	3	5	4
Sargassum lapazeanum	3	5	3
Sargassum linearifolium	3	5	2
Sargassum macrocarpum	3	6	4
Sargassum marginatum	3	5	2
Sargassum micracanthum	3	6	4
Sargassum miyabei	**1**	**1**	**0**
Sargassum natans	3	5	2
Sargassum nigrifolium	3	6	4
Sargassum obovatum	3	6	2
Sargassum obovatum var. pfeifferae	3	5	2
Sargassum obovatum var. robillardii	2	6	2
Sargassum obtusifolium	3	5	2
Sargassum pacificum	3	5	2
Sargassum paradoxum	3	6	4
Sargassum patens	3	6	3
Sargassum piluliferum	3	5	2

Species 13: Cordylophora caspia

Full name: *Cordylophora caspia* (Pallas, 1771)

The genus *Cordylophora* comprises three valid species *Cordylophora caspia* (Pallas, 1771), *Cordylophora japonica* Itô, 1951 and *Cordylophora solangiae* Redier, 1967 (WoRMS Editorial Board 2015). Only nucleotide sequence data for *Cordylophora caspia* is available on NCBI GenBank. The comparison between sequences from closely related non-target species is therefore based on nucleotide sequence data from other representatives of the family Oceaniidae. Without sequence information available from *C. japonica* and *C. solangiae* it is currently not possible to determine whether the recommend primer-probe system for detection of eDNA from *Cordylophora caspia* will be able to differentiate between *C. japonica* and *C. solangiae* and *Cordylophora caspia*.

The recommended primer-probe system for *Cordylophora caspia* should be able to detect eDNA originating from the mtDNA cytochrome oxidae 1 gene (Table S13.1).

- Cor_cas_CO1_F01: 5'-TCATCTGTACAAGCACATTCTGG-3',
- Cor_cas_CO1_R01: 5'-TTGAAGAAGCTCCTGCACAGT-3',
- Cor_cas_CO1_P01: 5'-FAM-CCTTCTGTAGACATGGCTATATTTAGTC-BHQ1-3',

Table S13.1: *Species specific primer/probe assay for* Cordylophora caspia *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	Gene	Product size	Temp	Length	GC
Cordylophora caspia	CO1	76 bp			
Cor_cas_CO1_F01	TCATCTGTACAAGCACATTCTGG		60.2	23	43
Cor_cas_CO1_R01	TTGAAGAAGCTCCTGCACAGT		60.2	21	48
Cor_cas_CO1_P01	CCTTCTGTAGACATGGCTATATTTAGTC		59.2	28	39

Related species	Forward	Reverse	Probe
Cordylophora sp. NFR1	0	0	0
Cordylophora sp. NFR9	4	2	6
Turritopsis lata	9	4	7
Turritopsis nutricula	9	4	7
Turritopsis rubra	6	3	6

An additional primer-probe system for eDNA from the nuclear 28S region is recommended in case the first recommended system turns out to be unable to distinguish *C. japonica* and *C. solangiae* from *Cordylophora caspia* (Table S13.2):

- Cor_cas_28S_F02: 5'-ACTGGACAGAGGAGGAGTCG-3',
- Cor_cas_28S_R02: 5'-CGACGACCAACAGTGACAAG-3',
- Cor_cas_28S_P02: 5'-FAM-ACATGCTCTTTTGGGCTGGCCTCT-BHQ1-3',

Table S13.2: *Species specific primer/probe assay for* Cordylophora caspia *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	Gene	Product size	Temp	Length	GC
Cordylophora caspia	28S	70 bp			
Cor_cas_28S_F02	ACTGGACAGAGGAGGAGTCG		59.4	20	60
Cor_cas_28S_R02	CGACGACCAACAGTGACAAG		60.4	20	55
Cor_cas_28S_P02	ACATGCTCTTTTGGGCTGGCCTCT		70.1	24	54

Related species	Forward	Reverse	Probe
Turritopsis dohrnii	9	9	5
Turritopsis lata	9	9	5
Turritopsis rubra	9	9	4

Species 14: Styela clava

Full name: *Styela clava* Herdman, 1881

The genus *Styela* comprises more than 200 species worldwide (WoRMS Editorial Board 2015), and around 18 species of *Styela* can be found in European seas (Monniot 2001), including *Styela clava*. A phylogenetic study on the families Pyuridae and Styelidae (Pérez-Portela *et al.* 2009) found a close relationship between *Styela montereyensis*, *S. gibbsii*, *S. plicata* and *Styela clava*. *Styela plicata* is occurring in the Atlantic and Mediterraen Sea, and could therefore lead to false positive eDNA detection.

Because very few species of the 244 species of *Styela* (WoRMS Editorial Board 2015) have nucleotide sequence data deposited on the NCBI GenBank database (Nov-2015), it is currently impossible to rule out that the suggested primer and probe systems for detection of mitochondrial eDNA fragments from *Styela clava* potentially could give rise to false positive eDNA detection because of nucleotide similarity with other co-occurring species of *Styela*.

In an attempt to minimize this risk of obtaining false positive eDNA detection due to other co-occurring non-sequenced species of *Styela*, a second primer-probe system for eDNA detection from mitochondrial cytochrome oxidase 1 gene fragments of *S. clava* is here recommended used together with the first recommended systems that targets eDNA from the mitochondrial cytochrome b region. Hopefully, the combination of these two primer-probe systems used together will reduce the number of false positives that might arise due to similarity in mitochondrial gene regions from the non-sequenced closely related species of *Stylea*. Because there appears to limited variation among mitochondrial haplotypes among *S. clava* (Goldstien *et al.* 2011), there might be a risk of not detecting haplotypes that are deviating from the most common haplotype among *S. clava* (Goldstien *et al.* 2011). Here two different species-specific systems are recommended for targeting eDNA fragments from mitochondrial cytochrome oxidase 1 from *S. clava* (Table S14.1, Table S14.2).

- Sty_cla_Cytb_F01: 5'-GAGTTTTTGAGGGGCAACTG-3',
- Sty_cla_Cytb_R01: 5'-TGGCCAACTGAAAACCTACC-3',
- Sty_cla_Cytb_P01: 5'-FAM-TGAGTGTAATCCCTTTTTATGGGGTGG-BHQ-1-3',

Table S14.1: *Species-specific primer/probe assay for* Styela clava *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	Gene	Product size	Temp	Length	GC
Styela clava	Cytb	102 bp			
Sty_cla_Cytb_F01	GAGTTTTTGAGGGGCAACTG		59.7	20	50
Sty_cla_Cytb_R01	TGGCCAACTGAAAACCTACC		60.0	20	50
Sty_cla_Cytb_P01	TGAGTGTAATCCCTTTTTATGGGGTGG		67.5	27	44

Related species	Forward	Reverse	Probe
Botrylloides leachii	5	12	6
Botrylloides nigrum	4	8	6
Botrylloides pizoni	4	9	7
Botrylloides violaceus	4	9	10
Botryllus planus	4	9	11
Cnemidocarpa finmarkiensis	2	8	9
Dendrodoa grossularia	3	9	6
Metandrocarpa taylori	4	8	12
Polycarpa mytiligera	4	7	6
Polycarpa papillata	2	7	8
Styela clava	0	0	0
Styela gibbsii	6	9	8

Styela montereyensis	4	8	7
Styela plicata	4	7	8
Symplegma viride	4	7	8

- Sty_cla_CO1_F02: 5'-GTAATTGTTACGGCCCATGC-3',
- Sty_cla_CO1_R02: 5'-CGGACTTCCCAACATCAAAG-3',
- Sty_cla_CO1_P02: 5'-FAM-TGCCGGTAATAATTAGAAGATTTAGGA-BHQ1-3',

Table S14.2: *Species-specific primer/probe assay for* Styela clava *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Styela clava	CO1	105 bp			
Sty_cla_CO1_F02	GTAATTGTTACGGCCCATGC		60.2	20	50
Sty_cla_CO1_R02	CGGACTTCCCAACATCAAAG		60.5	20	50
Sty_cla_CO1_P02	TGCCGGTAATAATTAGAAGATTTAGGA		61.5	27	33

Related species	**Forward**	**Reverse**	**Probe**
Boltenia ovifera	5	7	8
Botrylloides fuscus	4	8	7
Botrylloides leachii	4	6	10
Botrylloides nigrum	5	3	5
Botrylloides perspicuus	1	5	8
Botrylloides pizoni	1	5	8
Botrylloides violaceus	2	5	8
Botryllus schlosseri	5	4	6
Botryllus tyreus	4	5	6
Dendrodoa grossularia	4	6	6
Distomus variolosus	4	8	6
Halocynthia papillosa	4	8	9
Halocynthia pyriformis	3	9	5
Herdmania grandis	5	6	9
Microcosmus claudicans	4	3	5
Microcosmus polymorphus	1	5	10
Microcosmus squamiger	4	5	7
Polyandrocarpa zorritensis	3	5	9
Polycarpa aurata	4	4	9
Polycarpa pomaria	5	5	9
Polycarpa tenera	5	5	9
Polyzoa opuntia	5	6	10
Pyura australis	5	5	9
Pyura dura	3	3	7
Pyura gibbosa	5	4	9
Pyura praeputialis	5	4	7
Pyura spinifera	3	2	9
Pyura squamulosa	5	3	7
Styela canopus	3	5	8
Styela clava	0	0	0
Styela gibbsii	3	5	6
Styela montereyensis	3	5	5
Styela plicata	5	5	7
Symplegma rubra	3	10	7

Species 15: Dreissena polymorpha

Full name: *Dreissena polymorpha* (Pallas, 1771)

According to the Worms database (WoRMS Editorial Board 2015) the genus *Dreissena* comprises 11 species: *Dreissena anatolica* Locard, 1893, *D. bourguignati* Locard, 1883, *D. bugensis* (Andrusov, 1897), *D. caputlacus* Schütt, 1993, *D. carinata* (Dunker, 1853), *D. caspia* Eichwald, 1855, *D. elata* Andrusov, 1897, *D. gallandi* Locard, 1893, *D. polymorpha* (Pallas, 1771), *D. rostriformis* (Deshayes, 1838) and *D. siouffi* Locard, 1893. From the NCBI GenBank database five species of *Dreissena* have nucleotide sequence information deposited, including *D. polymorpha. Dreissena polymorpha* is native to the Caspian Sea (Kantor *et al.* 2010).

The nucleotide sequence comparisons made here are made with the five species of *Dreissena* represented on NCBI GenBank: *Dreissena blanci*, *D. caputlacus*, *D. polymorpha*, *D. presbensis*, and *D. rostriformis*. The nucleotide sequence comparisons have been made with representatives of Dreissenidae and the recommended primer-probe systems may very well be unable differentiate between *D. polymorpha* and other species of *Dreissena* not represented on NCBI GenBank. Additional sampling and sequencing of other species of *Dreissena* would be required to verify whether these suggested primer- probe system are species-specific (Table S15.1, Table S15.2, Table S15.3).

- Dre_pol_CO1_F01: 5'-CACCACTCATGGGCTTGTTA-3',
- Dre_pol_CO1_R01: 5'-ACCAATCAATTTCCGAATCC-3',
- Dre_pol_CO1_P01: 5'-FAM-TTTTTCTAGTAATACCTATAATAATGGGG-BHQ-1-3',

Table S15.1: *Species-specific primer/probe assay for* Dreissena polymorpha *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	Gene	Product size	Temp	Length	GC
Dreissena polymorpha polymorpha	CO1	75 bp			
Dre_pol_CO1_F01	CACCACTCATGGGCTTGTTA		59.6	20	50
Dre_pol_CO1_R01	ACCAATCAATTTCCGAATCC		58.7	20	40
Dre_pol_CO1_P01	TTTTTCTAGTAATACCTATAATAATGGGG		57.3	29	28

Related species	Forward	Reverse	Probe
Dreissena blanci	4	2	4
Dreissena polymorpha anatolica	0	0	1
Dreissena polymorpha gallandi	0	0	0
Dreissena polymorpha polymorpha	0	0	0
Dreissena presbensis	3	3	4

- Dre_pol_CO1_F02: 5'-TCAGCTTTTAGGGAAGGAGGA-3',
- Dre_pol_CO1_R02: 5'-GCAGGGCCTGAATGTCCTAT-3',
- Dre_pol_CO1_P02: 5'-FAM-GGGGGTGGTTGAACCTTATATCCTCCT–BHQ-1-3',

Table S15.2: *Species-specific primer/probe assay for* Dreissena polymorpha *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	Gene	Product size	Temp	Length	GC
Dreissena polymorpha polymorpha	CO1	83 bp			
Dre_pol_CO1_F02	TCAGCTTTTAGGGAAGGAGGA		60.3	21	48
Dre_pol_CO1_R02	GCAGGGCCTGAATGTCCTAT		61.4	20	55
Dre_pol_CO1_P02	GGGGGTGGTTGAACCTTATATCCTCCT		68.6	27	52

Related species	Forward	Reverse	Probe
Congeria jalzici	5	3	7
Congeria kusceri	4	3	6

Congeria mulaomerovici	5	4	6
Dreissena blanci	3	3	4
Dreissena caputlacus	6	2	5
Dreissena polymorpha	0	1	0
Dreissena polymorpha anatolica	0	1	0
Dreissena polymorpha anatolica	0	1	0
Dreissena polymorpha gallandi	0	1	1
Dreissena polymorpha polymorpha	0	0	0
Dreissena presbensis	2	3	3
Dreissena rostriformis	5	4	5
Dreissena rostriformis bugensis	5	3	5
Mytilopsis sallei	3	4	3

- Dre_pol_CO1_F03: 5'-GGTCCCTTTGTTCTGCACAT-3',
- Dre_pol_CO1_R03: 5'-AAAGCCCCAGCTAATACAGG-3',
- Dre_pol_CO1_P03: 5'-FAM-TGGTGTGACCAGATTCCTTTTAATCTT–BHQ-1-3',

Table S15.3: *Species-specific primer/probe assay for* Dreissena polymorpha *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Dreissena polymorpha polymorpha	CO1	78 bp			
Dre_pol_CO1_F03	GGTCCCTTTGTTCTGCACAT		60.0	20	50
Dre_pol_CO1_R03	AAAGCCCCAGCTAATACAGG		58.4	20	50
Dre_pol_CO1_P03	TGGTGTGACCAGATTCCTTTTAATCTT		64.1	27	37

Related species	**Forward**	**Reverse**	**Probe**
Congeria jalzici	5	3	6
Congeria kusceri	6	3	7
Congeria mulaomerovici	4	3	7
Dreissena blanci	3	3	6
Dreissena caputlacus	4	4	5
Dreissena polymorpha	0	1	0
Dreissena polymorpha anatolica	1	0	0
Dreissena polymorpha anatolica	1	0	0
Dreissena polymorpha gallandi	0	0	0
Dreissena polymorpha polymorpha	0	0	0
Dreissena presbensis	3	3	6
Dreissena rostriformis	4	5	5
Dreissena rostriformis bugensis	0	0	0
Mytilopsis sallei	0	0	0

Acipenser

It was not always possible to design species-specific primer-probe system. For example the sturgeons in the genus *Acipenser* comprises 17 species. *Acipenser baerii*, *A. gueldenstadtii*, *A. ruthenus* and *A. stellatus* are introduced in the Baltic whereas *A. sturio and A. oxyrinchus are* considered as native (Zhang *et al.* 2013, Froese & Pauly 2015). Hybridization is common among species of sturgeons (Tranah *et al.* 2004, Zhang *et al.* 2013) including *A. ruthenus*, *A. baerii*, *A. gueldenstaedtii* and *A. stellatus* (Zhang *et al.* 2013). This makes it very hard to develop functional a species-specific assay. Because species of *Acipenser* hybridize regularly it is considered to be impossible to develop a generally applicable single primer-probe assay that will be able to detect eDNA from all the various mitochondrial haplotypes in the different species of *Acipenser*. Instead primer-probe assays specific for unique haplotypes from the different targeted species of *Acipenser* are recommended. The endangered and native *A. sturio* and the *A. oxyrinchus* do not appear to hybridize with *A. baerii*, *A. gueldenstadtii*, *A. ruthenus* and *A. stellatus*, and the recommended eDNA detection system should therefore be able to distinguish native species (*A. oxyrinchus* and *A. sturio)* from introduced species (*A. baerii*, *A. gueldenstadtii*, *A. ruthenus* and *A. stellatus*).

Species 16: Acipenser baerii

Full name: *Acipenser baerii* Brandt, 1869

The recommended system targeting eDNA fragments from *A. baerii* might not be able to detect all haplotype variants of *A. baerii*, and might also return false positive amplification for other species of *Acipenser* carrying an *A. baerii* haplotype variant.

- Aci_bae_CR_F01: 5'-AGAGCCGAACATTCTTGTCTG-3',
- Aci_bae_CR_R01: 5'-TGGACCTGAAATAGGAACCAG-3',
- Aci_bae_CR_P01: 5'-FAM-TGAAGGACAGTAATTGTAGAGTTTCA–BHQ-1-3',

Table S16: *Species-specific primer/probe assay for* Acipenser baerii *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	Gene	Product size	Temp	Length	GC
Acipenser baerii	CR	112 bp			
Aci_bae_CR_F01	AGAGCCGAACATTCTTGTCTG		59.5	21	48
Aci_bae_CR_R01	TGGACCTGAAATAGGAACCAG		59.0	21	48
Aci_bae_CR_P01	TGAAGGACAGTAATTGTAGAGTTTCA		58.5	26	35

Related species	Forward	Reverse	Probe
Acipenser baerii baicalensis	13	11	18
Acipenser brevirostrum	14	10	18
Acipenser dabryanus	11	3	7
Acipenser fulvescens	2	0	4
Acipenser gueldenstaedtii	0	0	4
Acipenser medirostris	4	0	6
Acipenser mikadoi	13	12	17
Acipenser naccarii	0	0	4
Acipenser nudiventris	6	1	9
Acipenser oxyrinchus	18	6	20
Acipenser oxyrinchus desotoi	14	12	16
Acipenser persicus	0	1	5
Acipenser ruthenus	6	0	3
Acipenser schrenckii	6	0	4
Acipenser sinensis	13	12	17
Acipenser stellatus	13	11	17
Acipenser stellatus donensis	9	12	17
Acipenser stellatus stellatus	9	12	17
Acipenser sturio	14	10	16
Acipenser transmontanus	6	0	4
Polyodon spathula	7	0	6
Psephurus gladius	9	1	9
Scaphirhynchus albus	7	1	9
Scaphirhynchus platorynchus	7	1	9
Scaphirhynchus suttkusi	7	1	9

Species 17: Acipenser gueldenstaedtii

Full name: *Acipenser gueldenstaedtii* Brandt & Ratzeburg, 1833

For *Acipenser gueldenstaedtii* a species-specific assay for one haplotype of *A. gueldenstaedtii* is recommended. Note that this might not cover all haplotypes of *A. gueldenstaedtii*, and might also yield false positives from hybrids of *Acipenser*, including *A. baerii* (Table S17).

- Aci_gue_CR_F01: 5'-GAGCAGTCGTGAATGTTCCA-3',
- Aci_gue_CR_R01: 5'-CGAGAACCCCATCAACATTT-3',
- Aci_gue_CR_P01: 5'-FAM-ATCTTTTCTGAAGGAGCCCTGCATGT-BHQ-1-3',

Table S17: *Species-specific primer/probe assay for* Acipenser gueldenstaedtii *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Acipenser gueldenstaedtii	CR	112 bp			
Aci_gue_CR_F01	GAGCAGTCGTGAATGTTCCA		59.5	20	48
Aci_gue_CR_R01	CGAGAACCCCATCAACATTT		59.0	20	48
Aci_gue_CR_P01	ATCTTTTCTGAAGGAGCCCTGCATGT		65.0	26	38

Related species	**Forward**	**Reverse**	**Probe**
Acipenser baerii	0	0	0
Acipenser naccarii	5	1	4
Acipenser persicus	4	2	2
Acipenser ruthenus	2	2	7
Acipenser stellatus	6	3	14
Huso huso	4	1	4

Species 18: Acipenser ruthenus

Full name: *Acipenser ruthenus* Linnaeus, 1758

For *Acipenser ruthenus* a species-specific assay for one haplotype of *A. ruthenus* is recommended (Table S18). Note that this might not cover all haplotypes of *A. ruthenus*, and might also yield false positives from hybrids of *Acipenser*.

- Aci_rut_CR_F01: 5'-TGCCGAGAACCATATCAACA-3',
- Aci_rut_CR_R01: 5'-GACCGTGAATGTTCCACCTT-3',
- Aci_rut_CR_P01: 5'-FAM-ATTTGGGTTTCCATTCACTGACATGC-BHQ-1-3',

Table S18: *Species-specific primer/probe assay for* Acipenser ruthenus *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	Gene	Product size	Temp	Length	GC
Acipenser ruthenus	CR	112 bp			
Aci_rut_CR_F01	TGCCGAGAACCATATCAACA		60.1	20	45
Aci_rut_CR_R01	GACCGTGAATGTTCCACCTT		59.8	20	50
Aci_rut_CR_P01	ATTTGGGTTTCCATTCACTGACATGC		67.9	26	42

Related species	Forward	Reverse	Probe
Acipenser baerii	2	5	3
Acipenser baerii baicalensis	11	14	20
Acipenser baerii stenorrhynchus	11	13	20
Acipenser brevirostrum	13	13	8
Acipenser dabryanus	4	7	2
Acipenser fulvescens	3	6	4
Acipenser gueldenstaedtii	4	7	3
Acipenser gueldenstaedtii colchicus	13	13	18
Acipenser gueldenstaedtii gueldenstaedtii	13	13	19
Acipenser medirostris	4	6	2
Acipenser mikadoi	10	12	8
Acipenser naccarii	4	7	3
Acipenser nudiventris	4	5	2
Acipenser oxyrinchus desotoi	12	13	7
Acipenser persicus	3	7	3
Acipenser persicus persicus	13	13	19
Acipenser ruthenus	0	0	0
Acipenser schrenckii	5	5	2
Acipenser sinensis	4	7	2
Acipenser stellatus	12	13	8
Acipenser stellatus donensis	11	13	20
Acipenser stellatus ponticus	12	12	20
Acipenser stellatus stellatus	12	13	20
Acipenser sturio	9	14	8
Acipenser transmontanus	4	5	2
Huso huso	12	12	18
Huso huso caspicus	12	12	18
Huso huso maeoticus	12	12	18
Polyodon spathula	4	9	5
Scaphirhynchus albus	2	8	3
Scaphirhynchus platorynchus	2	7	3
Scaphirhynchus suttkusi	2	7	3

Species 19: Acipenser stellatus

Full name: *Acipenser stellatus* Pallas, 1771

For *Acipenser stellatus* a species-specific assay for one haplotype of *A. stellatus* is recommended (Table S19). Note that this might not cover all haplotypes of *A. stellatus*, and might also yield false positives from hybrids of *Acipenser*.

- Aci_ste_CR_F03: 5'-GTGTGAGGGAGAAAAGACCA-3',
- Aci_ste_CR_R03: 5'-CACCACATACAAACCTGCGTTA-3',
- Aci_ste_CR_P03: 5'-FAM-GGCCTCGTGAAAACACTCCTCATGT-BHQ-1-3'

Table S19: *Species-specific primer/probe assay for* Acipenser stellatus *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Acipenser stellatus stellatus	CR	70 bp			
Aci_ste_CR_F03	GTGTGAGGGAGAAAAGACCA		60.1	20	50
Aci_ste_CR_R03	CACCACATACAAACCTGCGTTA		60.8	22	45
Aci_ste_CR_P03	GGCCTCGTGAAAACACTCCTCATGT		68.6	25	52

Related species	**Forward**	**Reverse**	**Probe**
Acipenser baerii	8	13	3
Acipenser brevirostrum	13	17	18
Acipenser fulvescens	17	15	13
Acipenser fulvescens	17	15	13
Acipenser gueldenstaedtii	13	17	18
Acipenser medirostris	17	16	17
Acipenser medirostris	17	16	17
Acipenser mikadoi	13	17	18
Acipenser naccarii	17	15	17
Acipenser naccarii	17	15	17
Acipenser naccarii	17	15	17
Acipenser nudiventris	12	16	19
Acipenser oxyrinchus	17	16	15
Acipenser persicus	17	15	15
Acipenser persicus persicus	8	10	7
Acipenser ruthenus	17	16	17
Acipenser schrenckii	13	19	18
Acipenser sinensis	13	17	19
Acipenser stellatus	17	16	16
Acipenser stellatus stellatus	0	0	0
Acipenser sturio	13	17	18
Acipenser transmontanus	13	17	18
Huso huso	8	9	5

Species 20: Huso huso

Full name: *Huso huso* (Linnaeus, 1758)

The genus *Huso* comprises two species *H. huso* (Linnaeus, 1758) and *H. dauricus* (Georgi, 1775). For *Huso huso* a species-specific assay is recommended (Table S20) that should be able to distinguish between *Huso* and *Acipenser*. Further validation tests would be required to test whether this system can distinguish between *H. huso* and *H. dauricus*. *Huso huso* is native to Eurasia, the Caspian Sea and Black Sea and *H. dauricus* is native to Eastern Asia, and the recommended system will likely detect both invasive species (i.e. *H. huso* and *H. dauricus*), but note that the system might be unable to distinguish between these two species.

- Hus_hus_Cytb_F01: 5'-TCCGATCCATCCCAAATAAA-3',
- Hus_hus_Cytb_R01: 5'-TTCCCCGTTGTTTAGAGGTG-3',
- Hus_hus_Cytb_P01: 5'-FAM-GGCGGGGTACTAGCCCTTCTATTCTC-BHQ1-3',

Table S20: *Species-specific primer/probe assay for* Huso huso *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Huso huso	Cytb	102 bp			
Hus_hus_Cytb_F01	TCCGATCCATCCCAAATAAA		60.1	20	40
Hus_hus_Cytb_R01	TTCCCCGTTGTTTAGAGGTG		60.0	20	50
Hus_hus_Cytb_P01	GGCGGGGTACTAGCCCTTCTATTCTC		67.8	26	58

Related species	**Forward**	**Reverse**	**Probe**
Acipenser baerii	3	0	2
Acipenser brevirostrum	2	1	2
Acipenser dabryanus	2	0	1
Acipenser dabryanus	2	0	1
Acipenser fulvescens	3	0	2
Acipenser gueldenstaedtii	3	0	2
Acipenser medirostris	1	1	1
Acipenser mikadoi	1	1	0
Acipenser naccarii	3	0	2
Acipenser nudiventris	2	0	3
Acipenser oxyrinchus	3	1	1
Acipenser persicus	3	0	2
Acipenser ruthenus	3	1	4
Acipenser schrenckii	1	0	1
Acipenser sinensis	2	0	1
Acipenser stellatus	2	1	3
Acipenser sturio	3	2	1
Acipenser transmontanus	2	0	2
Huso huso	0	0	0
Polyodon spathula	3	1	5
Psephurus gladius	4	0	3

Species 21: Oncorhynchus gorbuscha

Full name: *Oncorhynchus gorbuscha* (Walbaum, 1861)

The genus *Oncorhynchus* belongs to the family Salmonidae and contains 15 species (Froese & Pauly 2015).

The following primers and probes are designed to target *Oncorhynchus gorbuscha:*

- Onc_Gor_Cytb_F01: 5'-CAGGAGCATCCGTCGACTT-3'
- Onc_Gor _Cytb_R01: 5'-CTGAGAGATTGCCGGTGGTT-3'
- Onc_Gor _Cytb_P01: 5'-FAM-GCTGGAATCTCATCAATTTTAGGGGCCA-BHQ-1-3'

Both *O. gorbuscha* and its sister-species are well represented by sequence material. Only for the single sister-species *O. rhodurus* is sequence not available. However, according Froese and Pauly (2015) this species is endemic to lake Biwa, Chûzenji and Ashinoko in Japan and is therefore not considered to pose a threat of false positives. Additional to the BLAST search of the assay, two native Danish salmonid species were included in the comparison to strengthen the precision of the assay, see Table S21.

Table S21: *Species-specific primer/probe assay for* Oncorchynchus gorbuscha *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Oncorhynchus mykiss	CytB	119 bp			
Onc_Gor_Cytb_F01	CAGGAGCATCCGTCGACTT		59.5	19	58
Onc_Gor _Cytb_R01	CTGAGAGATTGCCGGTGGTT		60.0	20	55
Onc_Gor _Cytb_P01	GCTGGAATCTCATCAATTTTAGGGGCCA		65.3	28	46

Related species	**F01**	**R01**	**P01**
Oncorhynchus mykiss	4	3	2
Oncorhynchus mykiss x *Salmo salar_KP218514*	4	3	2
Oncorhynchus mykiss x *Salmo salar_NC_026537*	4	3	2
Onchorhynchus gilae	4	3	3
Oncorhynchus chrysogaster	4	3	2
Oncorhynchus keta	2	4	2
Oncorhynchus clarkii	4	3	4
Oncorhynchus nerka	4	4	3
Oncorhynchus tshawytscha	5	2	4
Oncorhynchus kisutch	5	4	4
Oncorhynchus masou	4	5	3
Salmo trutta	2	5	4
Salmo salar	2	3	5

Species 22: Salvinus fontinalis

Full name: *Salvinus fontinalis* Mitchill, 1814.

The genus Salvinus belongs to the family Salmonidae and is comprised of nine marine species (WoRMS 2015). Sequences for target and sister-species were well represented in the NCBI database.

An assay for *S. fontinalis* has already been published and validated by Jane *et al.* (2014) based on the cytochrome b region. The assay utilizes a TaqMan MGB probe:

- Sal_For_Cytb_F01: 5'-CCATGAGGGCAAATATCCTTCTGA-3'
- Sal_For_Cytb_R01: 5'-TCATTGTACAAGGGCACCTCCTA-3'
- Sal_For_Cytb_P01: 5'-FAM-CTCCTCTCTGCTGTACCC-MGB-NFQ-3'

The annealing temperature utilized for this assay is 60 °C, whereas the values in table S22.1 are the temperatures calculated in Geneious version 9.0.4. Due to a low number of mismatches between *S. alpinus* and *S.* malma and the utilization of a TaqMan MGB probe, a secondary assay was also designed to improve precision.

Table S22.1: *Species-specific primer/probe assay for* Salvinus fontinalis *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Salvinus fontinalis	Cytb	90bp			
Sal_For_Cytb_F01	CCATGAGGGCAAATATCCTTCTGA		60.4	24	45.8
Sal_For_Cytb_R01	TCATTGTACAAGGGCACCTCCTA		61.1	23	47.8
Sal_For_Cytb_P01	CTCCTCTCTGCTGTACCC*		55.7	18	61.1

*TaqMan MGB probe

Related species	**F01**	**R01**	**P01**
Salvelinus albus	2	0	3
Salvelinus malma	1	0	3
Salvelinus taranetzi	2	0	3
Salvelinus alpinus	0	0	3
Salvelinus confluentus	2	0	3
Salvelinus namaycush	2	1	4
Salvelinus leucomaenis	1	1	4
Salvelinus malma malma × *Salvelinus leucomaenis*	1	0	4
Salvelinus levanidovi	4	1	4

*MGB probe

The following primers and probe are designed to target *Salvelinus fontinalis*:

- Sal_For_Cytb_F01: 5'-AGGGCAAATATCCTTCTGAGGG-3'
- Sal_For_Cytb_R01: 5'-GGCACCTCCTACATAGGGTAC-3'
- Sal_For_Cytb_P01: 5'-FAM-GCCACTGTAATTACAAACCTCCTCTCTGC-BHQ-1-3'

Table S22.2: *Species-specific primer/probe assay for* Salvinus fontinalis *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Salvinus fontinalis	Cytb	73 bp			
Sal_For_Cytb_F02	AGGGCAAATATCCTTCTGAGGG		59.6	22	50
Sal_For_Cytb_R02	GGCACCTCCTACATAGGGTAC		59	21	57.1
Sal_For_Cytb_P02	GCCACTGTAATTACAAACCTCCTCTCTGC		65.5	29	48.3

Related species	**F02**	**R02**	**P02**
Salvelinus albus	3	2	3
Salvelinus malma	2	2	3
Salvelinus taranetzi	3	2	3
Salvelinus alpinus	2	2	3
Salvelinus confluentus	3	2	3
Salvelinus namaycush	3	2	3
Salvelinus leucomaenis	3	2	3
Salvelinus malma malma × *Salvelinus leucomaenis*	2	3	2
Salvelinus levanidovi	5	3	2

Species 23: Colpomenia peregrine

Full name: *Colpomenia peregrine* (Sauvageau) Hamel 1937

The genus *Colpomenia* contains 14 species and both *rbc*L and *psa*A genes have been sequenced, however not the *mat*K gene. The *psa*A gene does not show strong representation among the species in the genus and could therefore not be used for the design of a species specific assay for *Colpomenia peregrine*. The *rbc*L gene on the other hand has been more widely sequenced, however was found not useful for assay design. In addition, the COX3 gene was represented at a significant scale. After looking through the three genes COX3 was identified as the only gene were it was possible to design an assay containing enough differentiation in primer/probe sequences to non-target species, as seen in table S23:

- Col_per_COX3_F01: 5' GCAAGCTTTTGAATATGCTAATG-3'
- Col_per_COX3_R01: 5'-CAGCTAAAAATATTGTACCGATTA-3'
- Col_per_COX3_P01: 5'-FAM-TTCAGTTTTTTACATGGCTACAGGCTTC-BHQ-1-3'

Table S23: *Species specific primer/probe assay for* Colpomenia peregrina *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Colpomenia peregrina	COX3	116 bp			
Col_per_COX3_F01	GCAAGCTTTTGAATATGCTAATG		57.6	23	35
Col_per_COX3_R01	CAGCTAAAAATATTGTACCGATTA		56.6	24	29
Col_per_COX3_P01	TTCAGTTTTTTACATGGCTACAGGCTTC		65.6	28	39

Related species*	**Forward**	**Reverse**	**Probe**
Colpomenia bullosa	7	6	4
Colpomenia claytoniae	1	3	4
Colpomenia durvillei	7	3	4
Colpomenia ecuticulata	6	5	5
Colpomenia expansa	1	6	5
Colpomenia phaeodactyla	8	4	4
Colpomenia ramosa	7	5	7
Colpomenia sinuosa	4	6	6
Colpomenia tuberculata	7	5	7
Colpomenia sp. CCC-2009a	7	5	4
Colpomenia sp. KML-2014	8	4	4
Petalonia zosterifolia	7	5	2
Scytosiphon lomentaria	6	5	6
Scytosiphon sp. CCC-2009a	6	5	6
Desmarestia menziesii	9	5	4
Padina pavonica	8	7	5

* not necessarily related but show strong sequence similarity to target species

Species 24: Ensis directus

Full name: *Ensis directus* (Conrad, 1843)

Ensis americanus (Gould, 1870) is considered a junior synonym of *E. directus* (Cosel 2009). The genus *Ensis* comprises around 30 species (WoRMS Editorial Board 2015) and on the NCBI Genbank nucleotide sequences are available from nine species of *Ensis*, including *E. directus*. Since the NCBI GenBank database does not have all known species of *Ensis* represented, false positives from closely related non-target species is a risk. The primers and probe suggested here will only be able differentiate between species of *Ensis* that are represented by nucleotide sequences on the NCBI Genbank database.
A population study on *E. directus* and *E. terranovensis* Vierna & Martínez-Lage, 2012 found subtle differences in the genetic variation (Vierna *et al.* 2012).

For detection of eDNA from cytochrome oxidase 1 in the mitochondrial genome from *Ensis directus* the following primers and probes are recommended (Table S24.1 and Table S24.2):

- Ens_dir_CO1_F01: 5'- GTTTGTTGGTAGCGGGATTG -3',
- Ens_dir_CO1_R01: 5'- TCCCATAATCCATTGAAGGAC -3',
- Ens_dir_CO1_P01: 5'-FAM- GCCGGATGAACTATTTATCCGCCTTTA -BHQ-1-3'

Table S24.1: *Species specific primer/probe assay for* E. directus *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Ensis directus	mtDNA-CO1	92 bp			
Ens_dir_CO1_F01	GTTTGTTGGTAGCGGGATTG		60.4	20	50
Ens_dir_CO1_R01	TCCCATAATCCATTGAAGGAC		58.7	21	43
Ens_dir_CO1_P01	GCCGGATGAACTATTTATCCGCCTTTA		68.3	27	44

Related species	**Forward**	**Reverse**	**Probe**
Ensis ensis	4	3	3
Ensis goreensis	4	4	5
Ensis macha	3	1	3
Ensis magnus	3	3	5
Ensis minor	3	2	4
Ensis minor	3	2	4
Ensis siliqua	3	4	2
Ensis terranovensis	2	0	2
Phaxas pellucidus	4	3	2

An additional system for detection of eDNA from cytochrome oxidase 1 in the mitochondrial genome from *Ensis directus* is also recommended. In case the first one is unspecific:

- Ens_dir_CO1_F02: 5'- CATTTCCTCGATTAAACAATTTGA-3',
- Ens_dir_CO1_R02: 5'- CAATCCCGCTACCAACAAAC -3',
- Ens_dir_CO1_P02: 5'-FAM- TTGACTTTTACCTTGTGCTTTGTTTTT-BHQ-1-3'

Table S24.2: *Species specific primer/probe assay for* E. directus *with, target gene, product size, melting*

temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.

Species	Gene	Product size	Temp	Length	GC
Ensis directus	mtDNA-CO1	90 bp			
Ens_dir_CO1_F02	CATTTCCTCGATTAAACAATTTGA		59.4	24	29
Ens_dir_CO1_R02	CAATCCCGCTACCAACAAAC		60.4	20	50
Ens_dir_CO1_P02	TTGACTTTTACCTTGTGCTTTGTTTTT		62.4	27	30

Related species	Forward	Reverse	Probe
Ensis ensis	5	4	4
Ensis goreensis	5	4	7
Ensis macha	5	3	4
Ensis magnus	4	3	8
Ensis minor	2	3	1
Ensis minor	2	3	1
Ensis siliqua	4	3	7
Ensis terranovensis	1	2	2
Phaxas pellucidus	3	4	4

These two eDNA detection systems targeting *E. directus* might not be able to differentiate between *E. directus* and *Ensis terranovensis*. But as both *E. directus* and *Ensis terranovensis* are native inhabitants in the North Western Atlantic and both are considered introduced in the North Eastern Atlantic, this inability to differentiate between these two closely related species might be considered irrelevant for the purpose of monitoring the invasive *E. directus-E. terranovensis* species complex in European seas.

Species 25: Dasya baillouviana

Full name: *Dasya baillouviana* (Gmelin) Montagne, 1841

The genus *Dasya* represents 19 different species. For 12 of these species the *rbc*L gene has been sequenced, and due to the indicated species specificity of this gene for eDNA assay design for aquatic plants (Scriver *et al.* 2015), of available sequences genes for *Dasya baillouviana* and relevant non-target species, this gene was selected. For better assay design a subset of various species showing sequence similarity through a BLAST search were included in the sequence alignment (Table S25). Assay design in *rbc*L was not trivial and base pair mismatch to non-target species was maybe not as high as one could desire. Despite this a species specific eDNA primer/probe assay was designed for *Dasya baillouviana*:

- Das_bai_rbcL_F01: 5'-CAAACTTAACTGCATCTATCATC-3'
- Das_bai_rbcL_R01: 5'-AATCCTGTTGCAGGACCTTG-3'
- Das_bai_rbcL_P01: 5'-FAM-CGTATCCCAGTAGCTTATCTAAAAACATT-BHQ-1-3'

Table S25: *Species specific primer/probe assay for* Dasya baillouviana *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species*

Species	Gene	Product size	Temp	Length	GC
Dasya baillouviana	rbcL	113 bp			
Das_bai_rbcL_F01	CAAACTTAACTGCATCTATCATC		57.6	23	35
Das_bai_rbcL_R01	AATCCTGTTGCAGGACCTTG		58.4	20	50
Das_bai_rbcL_P01	CGTATCCCAGTAGCTTATCTAAAAACATT		64.6	29	34

Related species*	Forward	Reverse	Probe
Dasya anastomosans	4	1	5
Dasya collabens	4	2	3
Dasya elongata	4	1	6
Dasya enomotoi	2	3	4
Dasya hutchinsiae	4	2	4
Dasya iridescens	2	3	4
Dasya ocellata	3	1	2
Dasya sessilis	1	0	4
Dasya sp. GENT HV TZ13	4	1	5
Dasya sp. KS-2014	5	2	2
Dasya sp. L SGAD0712164	4	2	4
Dasyaceae sp. GH04	6	2	1
Heterosiphonia japonica	4	2	3
Dipterocladia pinnatifolia	4	2	6
Eupogodon planus	3	2	3
Rhodoptilum plumosum	3	0	1
Zellera tawallina	4	1	4
Chauviniella coriifolia	4	2	3
Branchioglossum woodii	4	2	3
Branchioglossum bipinnatifidum	5	3	3
Grinnellia americana	5	2	2
Chauviniella coriifolia	5	2	4
Phitymophora amansioides	5	3	4
Phitymorphora linearis	6	3	3
Hypoglossum hypoglossoides	4	3	4
Hypoglossum sp.1	4	2	4
Polyneuropsis stolonifera	5	4	3
Paraglossum crassinervium	4	2	2
Bartoniella crenata	6	2	1

* not necessarily related but show strong sequence similarity to target species

Species 26: Heterosiphonia japonica

Full name: *Heterosiphonia japonica* Yendo, 1920

The genus *Heterosiphonia* comprises 10 species. Despite relatively good representation of genes for assay design for *Heterosiphonia japonica* not all species within the genus were represented in all genes. Best representation and assay design was obtained using the *rbc*L gene:

- Het_jap_rbcL_F01: 5'- CAACAGGTATTATTGTAGAACGC-3'
- Het_jap_rbcL_R01: 5'-TAATTTTTACCAGATAGACCTAGC-3'
- Het_jap_rbcL_P01: 5'-FAM-ATAAGTTTGGCCGTCCTTTCCTAGGA-BHQ-1-3'

Mismatch to the genus *Dasya*, within the family *Dasyaceae* (within which the genus *Heterosiphonia* also belongs) was for *Dasya collabens* not possible to design, whereas another species on this list, *Dasya baillouviana*, did show any mismatches in the sequence regions of the assay to *Heterosiphonia japonica* (Table S26).

Table S26: *Species specific primer/probe assay for* Heterosiphonia japonica *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species. Problematic species are marked in bold*

Species	Gene	Product size	Temp	Length	GC
Heterosiphonia japonica	rbcL	99 bp			
Het_jap_rbcL_F01	CAACAGGTATTATTGTAGAACGC		59.2	23	39
Het_jap_rbcL_R01	TAATTTTTACCAGATAGACCTAGC		58.3	24	33
Het_jap_rbcL_P01	ATAAGTTTGGCCGTCCTTTCCTAGGA		66.2	26	46

Related species*	Forward	Reverse	Probe
Heterosiphonia gibbesii	4	3	8
Heterosiphonia plumosa	3	4	10
Heterosiphonia pulchra	3	5	8
Nannochloropsis gaditana	6	4	7
Achnanthidium saprophilum	3	4	12
Nitzschia amphibia	1	4	11
Coscinodiscus radiatus	3	4	9
Polysiphonia virgata	5	5	8
Polysiphonia denudata	4	5	7
Aulacoseira granulata	5	4	9
Rhodella violacea	7	4	6
Caloglossa rotundata	4	4	4
Eupogodon spinellus	2	3	4
Eupogodon planus	2	5	3
Rhodymenia corallina	5	4	3
Dasya sp. L SGAD0712164	**1**	**2**	**2**
Dasya collabens	**0**	**0**	**0**
Dasya baillouviana	5	4	5

* not necessarily related but show strong sequence similarity to target species

Species 27: Spartina anglica

Full name: *Spartina anglica*, C. E. Hubbard

According to the NCBI database the genus *Spartina* consists of 17 species. Within the genus sequence information was available for the *mat*K gene. However, alignment of the sequences revealed insufficient numbers of mismatches among the species, and it was therefore not possible to design a useful eDNA primer/probe assay for *Spartina anglica* using sequence information from this gene. Alignments of available *rbc*L sequence were all almost identical and therefore also not usable for assay design. Having tested the more recognised genes for eDNA primer/probe design (Scriver *et al.* 2015) alignment of available sequences for the *trn*T-*trn*L and *trn*L-*trn*F intergenic spacer region was tried, however also unsuccessful.

According to the UniProt consortium (The UniProt Consortium 2007) the species *Spartina anglica* is a synonym of *Sporobolus anglicus* (*C.E. Hubb.*) P.M. Peterson & Saarela. However, despite extending the search to include information for genes sequenced for *Sporobolus anglicus* (a genus containing 157 species) it was not possible to design an eDNA primer/probe assay for either of the two synonyms.

Species 28: Heterosigma akashiwo

Full name: *Heterosigma akashiwo* (Y. Hada) Y. Hada ex Y. Hara & M. Chihara, 1987

The genus *Heterosigma* comprises only this one species. The genus *Heterosigma* belongs to the family *Chattonellaceae* containing 18 species and variates of these.
An assay for *Heterosigma akashiwo* has already been published based on the 18S sequence (Coyne *et al.* 2005). However, this assay has a product size of 376bp. Due to this a new assay was designed based on available sequence information for the *rbc*L gene obtained from the NCBI online database:

- Het_aka_rbcL_F01: 5'-CTCTTGAAGCTATGATTTTAGCTA-3'
- Het_aka_rbcL_R01: 5'-CACATAATTTAGCGATGTCTCTT-3'
- Het_aka_rbcL_P01: 5'-FAM-CGAAGGTCGTGACTACGTTTCTGAAG-BHQ-1-3'

Species specificity and assay details can be seen in table S28. Target species was compared to most related species but also species showing sequence similarity identified by blasting the *rbc*L sequence for *Heterosigma akashiwo*. *Olisthodiscus luteus* (N. Carter) and *Fibrocapsa cf. japonica* (JX067601.1) were found to have identical sequence for *rbc*L as *Heterosigma akashiwo*. According to AlgaeBase.org *Olisthodiscus luteus* and *Heterosigma akashiwo* are most likely two different species. The sequence identity may therefore be caused by wrong species identification and registration within the NCBI online database for the gene. This could also be the reason why *Fibrocapsa cf. japonica* (AB280606.1) and *Heterosigma akashiwo* have identical sequences. The sequence available for *Chattonella minima* was not of equal length compared to the other species in the alignment and an alignment between this species and *Heterosigma akashiwo* could therefore not be done. However, comparison among the other *Chattonella* species in the alignment it is very likely that *Chattonella minima* would show the same pattern of mismatches as the others if sequenced at equal length. Because of this it is believed that the designed assay will not amplify for *Chattonella minima*.

Table S28: *Species specific primer/probe assay for* Heterosigma akashiwo *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species. Problematic species are marked in bold*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Heterosigma akashiwo	rbcL	99 bp			
Het_aka_rbcL_F01	CTCTTGAAGCTATGATTTTAGCTA		58.3	24	33
Het_aka_rbcL_R01	CACATAATTTAGCGATGTCTCTT		57.6	23	35
Het_aka_rbcL_P01	CGAAGGTCGTGACTACGTTTCTGAAG		67.9	26	50

Related species*	**F01**	**R01**	**P01**
Olisthodiscus luteus	0	0	0
Chattonella marian	7	4	5
C. minima	-	-	-
C. subsalsa	7	4	3
C. ovata	7	4	5
C. antiqua	7	4	5
Fibrocapsa japonica	2	5	5
Haramonas dimorpha	7	4	5
Ophiocytium capitatum	5	9	6
Ophiocytium majus	4	8	5
Tribonema regulare	5	8	5
Tribonema viride	5	8	5
Tribonema intermixum	5	8	5
Botrydium bechariarum	4	8	5
Bumilleria klebsiana	5	8	5
Xanthonema muciculum	7	8	4

* not necessarily related but show strong sequence similarity to target species

Species 29: Pseudochattonella farcimen

Full name: *Pseudochatonella farcimen* (W. Eikrem, B. Edvardsen & J. Throndsen) W. Eichrem, 2009

The genus *Pseudochattonella* only contains the two species *Pseudochattonella farcimen* and *Pseudochattonella verruculosa*. The two species are more or less impossible to tell apart morphologically after fixation in lygol. Due to this a molecular method has been developed and to date serve as the best solution to tell apart the two species (Dittami *et al.* 2013). However, the designed primers (best possible) only have two mismatches each, respectively. According to the published qPCR protocol it is relatively difficult even with the molecular method to determine if the amplification in the qPCR reaction is caused by the presence of the one or the other species (Dittami *et al.* 2013):

- PfarF: 5'-GAGGTCCCGGGGAGAAATT-3'
- PfarR: 5'-GCAACTCGACTCCACTAGG-3'

No other mismatches exist within the sequence between the two primers. Therefore, no probe was designed. However, it has come to our attention that a primer/probe assay is under development and awaiting publication (Eckford-Soper & Daugbjerg (in press)). With this new assay it is expected that differentiation between the two *Pseudochattonella* species will possible.

Table S29: *Species specific primer/probe assay for* Pseudochattonella farcimen *with, target gene, product size, melting temperature, primer/probe length, and GC ratio (%)*

Species	Gene	Product size	Temp	Length	GC
Pseudochattonella farcimen	28S	91 bp			
PfarF	GAGGTCCCGGGGAGAAATT		59.5	19	58
PfarR	GCAACTCGACTCCACTAGG		59.5	19	58

Species 30: Molgula manhattensis

Full name: *Molgula manhattensis* (De Kay, 1843)

According to WoRMS the genus *Molgula* comprises 143 marine species. The genus belongs to the family Molgulidae, which contains 15 genera (WoRMS 2015).

The most suitable genetic region was found to be 18S with the broadest interspecies coverage, representing 13 out of 143 species including the target species *M. manhattensis*. Based on the sequence data, there is a high level of species similarity, e.g. a pairwise alignment between *M. manhattensis* and *M. retortiformis* shows that only one nucleotide insertion separates the two species in a 964 nucleotide fragment. The limited interspecies variation and limited sequence material from sister-species leads to uncertainty with regards to assay specificity, depending on local species occurrences.

The following primers and probe are designed to target *M. manhattensis*:

- Mol_Man_18S_F01: 5'-CAAGGTGTGTACTGTCGGCC-3'
- Mol_Man_18S_R01: 5'-AAAGTAAACGCACTGGCCAC-3'
- Mol_Man_18S_P01: 5'-FAM-TCCGGCGGTTCTCTTAACTGAGTGTCG-BHQ-1-3'

Table S30: *Species-specific primer/probe assay for* Molgula manhattensis *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Molgula manhattensis	18S	90 bp			
Mol_Man_18S_F01	CAAGGTGTGTACTGTCGGCC		60.9	20	60
Mol_Man_18S_R01	AAAGTAAACGCACTGGCCAC		59.3	20	50
Mol_Man_18S_P01	TCCGGCGGTTCTCTTAACTGAGTGTCG		68.0	27	56

Related species	**F01**	**R01**	**P01**
Molgula retortiformis	0	0	0
Molgula arenata	0	0	2
Molgula provisionalis	0	0	2
Molgula socialis	0	0	2
Molgula complanata	3	5	5
Molgula tectiformis	4	6	4
Molgula occidentalis	2	2	4
Molgula bleizi	5	4	4
Molgula pugetiensis	5	3	4
Molgula oculata	4	4	4
Molgula pacifica	4	4	4
Molgula citrina	5	4	5

Species 31: Cercopagis pengoi

Full name: *Cercopagis pengoi* (Ostroumov, 1891)

Cercopagis pengoi is the only species within the genus *Cercopagis*. Within the Family *Cercopagididae* only one other genus (*Bythutrephes*) exists. Due to this, the sequence alignment search was extended to the infraorder *Onychopoda*. Within this infraorder all species sequenced for the CO1 gene were compiled for the design of the species specific eDNA primer/probe assay for *Cercopagis pengoi*. The assay was easily constructed and showed substantial mismatch to non-target species (table S31):

- Cer_pen_CO1_F01: 5'-ATTTTCTTCATGGTAATGCCAGTA-3'
- Cer_pen_CO1_R01: 5'-GGGAAGGAATCAGAAACTAAGA-3'
- Cer_pen_CO1_P01: 5'-FAM-AATTGACTTGTCCCTCTGATGCTAGG-BHQ-1-3'

Table S31: *Species specific primer/probe assay for* Cercopagis pengoi *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Cercopagis pengoi	CO1	122 bp			
Cer_pen_CO1_F01	ATTTTCTTCATGGTAATGCCAGTA		58.3	24	33
Cer_pen_CO1_R01	GGGAAGGAATCAGAAACTAAGA		58.4	22	41
Cer_pen_CO1_P01	AATTGACTTGTCCCTCTGATGCTAGG		66.2	26	46

Related species*	**Forward**	**Reverse**	**Probe**
Bythotrephes cederstroemi	9	3	6
Bythotrephes longimanus	8	3	6
Bythotrephes sp. BOLD:AAB9254	9	3	6
Cornigerius maeoticus	4	4	4
Evadne nordmanni	4	7	7
Evadne spinifera	3	3	7
Pleopis polyphemoides	4	6	7
Pleopis sp. BOLD:AAI2700	3	6	8
Podon intermedius	5	7	7
Podon leuckartii	7	4	7
Podonevadne angusta	5	5	5
Podonevadne camptonyx	5	5	5
Podonevadne trigona	5	5	5
Pseudevadne tergestina	3	5	6
Polyphemus pediculus	8	5	8
Polyphemus aff. pediculus 3 JRD-2015	6	5	7
Polyphemus pediculus complex sp. NA2	8	5	9
Polyphemus pediculus complex sp. NA3	6	5	7

* not necessarily related but show strong sequence similarity to target species

Species 32: Homarus americanus

Full name: *Homarus americanus*. Milne Edwards, 1837.

The genus *Homarus* comprises two marine species, *H. americanus* and *H. gammarus*. The genus belongs to the family Nephropidae which contains 16 genera with marine species (WoRMS 2015).

Both *H. americanus* and the sister-species *H. gammarus* are represented by sequence data. Additionally to the BLAST search of the assay, other decapod species were included in the alignment to improve and test the precision of the assay, see Table S32.

The following primers and probe are designed to target *H. americanus*:

- Hom_Ame_Cytb_F01: 5'-CCCTACCTTCTTGGAGACCC-3'
- Hom_Ame_Cytb_R01: 5'-CAGGTTGAATATGTGCTGGTGT-3'
- Hom_Ame_Cytb_P01: 5'-FAM-ACTTTGTCCCGGCTAATCCACTCGT-BHQ-1-3'

Table S32: *Species-specific primer/probe assay for* Homarus americanus *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Homarus americanus	Cytb	73 bp			
Hom_Ame_Cytb_F01	CCCTACCTTCTTGGAGACCC		58.8	20	60
Hom_Ame_Cytb_R01	CAGGTTGAATATGTGCTGGTGT		58.9	22	46
Hom_Ame_Cytb_P01	ACTTTGTCCCGGCTAATCCACTCGT		66	25	52

Related species	**F01**	**R01**	**P01**
Homarus gammerus	2	1	6
Nephrops norvegnicus	3	2	7
Eriocheir sinensis	5	6	9
Eriocheir japonica	4	7	9
Eriocheir hepuensis	4	5	9
Paralithodes camtschaticus	7	2	6

Species 33: Paralithodes camtschaticus

Full name: *Paralithodes camtschaticus* Tilesius, 1815.

The genus *Paralithodes* belongs to the family Lithodidae which contains ten genera. The genus *Paralithodes* has five marine species (WoRMS 2015).

Paralithodes camtschaticus sequence was compared to that of its most closely related species and to common native species and other non-native decapod species (following Muus *et al.* 2006). Sequence data was available from the NCBI database for three of the five congeners; no sequence was found for *P. californiensis* and *P. rathbuni.* CO1 was found to be the most suitable genetic region with the broadest interspecies coverage.

The following primers and probe are designed to target *P. camtschaticus*:

- Par_Cam_CO1_F01: 5'-GCAGGAGCATCAGTGGATTT-3'
- Par_Cam_CO1_R01: 5'-AGGTTATTCCTTGTGGACGTATAT-3'
- Par_Cam_CO1_P01: 5'-FAM-TGGCTGGAGTATCTTCTATTTTAGGGGCT-BHQ-1-3'

Table S33: *Species-specific primer/probe assay for* Paralithodes camtschaticus *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	Gene	Product size	Temp	Length	GC
Paralithodes camtschaticus	CO1	118bp			
Par_Cam_CO1_F01	GCAGGAGCATCAGTGGATTT		58.2	20	50
Par_Cam_CO1_R01	AGGTTATTCCTTGTGGACGTATAT		57.3	24	37.5
Par_Cam_CO1_P01	TGGCTGGAGTATCTTCTATTTTAGGGGCT		65.0	29	44.8

Related species	F01	R01	P01
Paralithodes platypus	1	5	3
Paralithodes brevipes	3	3	5
Maja squinado	4	8	6
Nephrops norvegnicus	6	6	6
Homarus americanus	5	7	6
Homarus gammarus	5	7	6
Palinurus mauritanicus	5	6	7

Species 34: Didemnum vexillum

Full name: *Didemnum vexillum* Romanow, 1989 .

The genus Didemnum belongs to the family Didemnidae. The genus contains 236 marine species according to WoRMS.

CO1 was found to be the most suitable region due to the broadest coverage across sister-taxa. However, out of the 236 marine species within the genus *Didemnum* there are only 11 species that are represented by sequence data in the NCBI database. Furthermore, for available sequence data there is an unusually high level of intraspecies variation. Especially the species *D. candidum, D. granulatum* and to a lesser extent *D. vexillum*, all of which have multiple individual repository samples, exhibit more than 60 mismatches when aligned to other sequences. Species misidentification is common for this genus as there are few morphological characteristics and a high level of variability within the relevant characters (Stefaniak *et al.* 2009). Although the majority of sequence data for *D. vexillum* show similarity, the uncertainty presented by both the potential misidentification within the genus and the lack of genetic data for 225 sister-species, suggest that the specificity of any assay could be suboptimal.

Species 35: Fucus evanescens

Full name: *Fucus evanescens* C. Agardh, 1820

There are 14 species and variates within the genus *Fucus*. For the species in question, *Fucus evanescens*, only three genes were abundant enough within the genus to be considered for the task at hand. However:

- 18S not suitable to design species specific eDNA primer/probe assy.
- 23S does not provide useful sequence mismatch among *Fucus evanescens*, *F. serratus*, and *F. gardneri*. For the remaining species assay design was prohibited due to the formation of secondary structures and self-annealing of the primers or probe.
- 26S did also not provide the necessary sequence mismatch between target and non-target species. At the one area where an assay potentially could have been positioned this was prevented by the formation of secondary structures and self-annealing of the primers or probe.

Because of this no eDNA primer/probe assy could be designed for *Fucus evanescens*.

Species 36: Petricolaria pholadiformis

Full name *Petricolaria pholadiformis* (Lamarck, 1818)

The genus *Petricolaria* comprises 7 species (WoRMS Editorial Board 2015). But only *Petricolaria pholadiformis* (Lamarck, 1818) and *Petricola lapicida* (Gmelin, 1791) from Petricolidae are represented by nucleotide sequence data on the NCBI GenBank database (Nov-2015).

The recommended primers and probes will likely detect eDNA from the nuclear 28s gene in *Petricolaria pholadiformis*, but whether it will detect eDNA from 28s from other species of *Petricolaria* is not possible to say without first obtaining additional sequence information from 28s genes from other representatives of *Petricolaria* (Table S36.1).

- Pet_pho_28S_F01: 5'-AATTCAGCCGGGTTGTCTTT-3',
- Pet_pho_28S_R01: 5'-AGAAAGTGCACCGGGTCTTC-3',
- Pet_pho_28S_P01: 5'-FAM-ATCGTCCCTTCTCGGAGGGAAGAC-BHQ-1-3'

Table S36.1: *Species specific primer/probe assay for* Petricolaria pholadiformis *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	Gene	Product size	Temp	Length	GC
Petricolaria pholadiformis	28S	104 bp			
Pet_pho_28S_F01	AATTCAGCCGGGTTGTCTTT		60.9	20	45
Pet_pho_28S_R01	AGAAAGTGCACCGGGTCTTC		61.6	20	55
Pet_pho_28S_P01	ATCGTCCCTTCTCGGAGGGAAGAC		68.9	24	58

Related species	Forward	Reverse	Probe
Petricola lapicida	4	2	6

In case the recommended primers and probes for the 28s region is unable to differentiate between *Petricolaria pholadiformis* and other species of *Petricolaria*, a second system (Table S36.2) of primers and probes for detection of the mitochondrial cytochrome oxidase 1 gene is also recommended. But without other species of *Petricolaria* than just *Petricolaria pholadiformis* in the NCBI Gen Bank database, it is not possible to rule out that these primers and probes might return positive detection on other species of *Petricolaria*. It would require additional sequencing of correctly identified vouchered specimens of the other six species of *Petricolaria*, to ascertain whether the recommended system truly is species-specific.

- Pet_pho_CO1_F02: 5'-GATCCGGTTTAATGGGTACG-3',
- Pet_pho_CO1_R02: 5'-AAAATAGTCCCCGGATGAGC-3',
- Pet_pho_CO1_P02: 5'-FAM-GCTTTCAGTGTGATTATTCGTATGGAA-BHQ-1-3'

Table S36.2: *Species specific primer/probe assay for* Petricolaria pholadiformis *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	Gene	Product size	Temp	Length	GC
Petricolaria pholadiformis	CO1	70 bp			
Pet_pho_ CO1_F01	GATCCGGTTTAATGGGTACG		59.2	20	50
Pet_pho_ CO1_R01	AAAATAGTCCCCGGATGAGC		60.3	20	50
Pet_pho_ CO1_P01	GCTTTCAGTGTGATTATTCGTATGGAA		63.5	24	37

Related species	Forward	Reverse	Probe
Petricola lapicida	6	8	6

Species 37: Perccottus glenii

Full name: *Perccottus glenii* Dybowski, 1877.

The genus *Perccottus* includes the target species *P. glenii* only (Froese & Pauly 2015; WoRMS 2015). The genus belongs to the family Odontobutidae, containing five genera with a total of 21 species (Froese & Pauly 2015).

Sequence data were obtained for both *P. glenii* and the most closely related species from sister genera. Sequence data was available for eight out of a total of 21 species within the family Odontobutidae. Cytochrome b was found to be the most suitable region with best interspecies coverage.

The following primers and probe are designed to target *P. glenii*:

- Per_Gle_Cytb_F01: 5'-AGACAATGCAACTCTTACCCGA-3'
- Per_Gle_Cytb_R01: 5'-CTGCCGGGTTATTTGAGCCT-3'
- Per_Gle_Cytb_P01: 5'-FAM-GTTCCCTTTTGTTATTGCAGCATTCACTGT-BHQ-1-3'

Table S37: *Species-specific primer/probe assay for* Perccottus glenii *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Perccottus glenii	Cytb	119 bp			
Per_Gle_Cytb_F01	AGACAATGCAACTCTTACCCGA		59.7	22	46
Per_Gle_Cytb_R01	CTGCCGGGTTATTTGAGCCT		60.4	20	60
Per_Gle_Cytb_P01	GTTCCCTTTTGTTATTGCAGCATTCACTGT		65.0	30	40

Related species	**F01**	**R01**	**P01**
Odontobutis yaluensis	3	4	12
Odontobutis potamophila	3	7	11
Odontobutis interrupta	2	4	11
Odontobutis platycephala	3	3	14
Odontobutis obscura	2	3	14
Odontobutis sinensis	2	4	11
Micropercops cinctus	2	5	13

Species 38: Elminius modestus

Full name: *Elminius modestus* Darwin, 1854.

The genus *Elminius* includes six marine species (WoRMS 2015). The genus belongs to the family Austrobalanidae, containing two subfamilies and eight genera with a total of 23 species.

Multiple genetic regions including 12S, 16S, 18S, 28S and CO1 were investigated, but all regions have limited sequence material available for closely related species, both on a genus and a family level. Based on the data available at NCBI the assay is specific, but false positives with sister-species occurring in Danish territorial waters cannot be excluded.

Following primers and probe have been designed to target *E. modestus*:

- Aus_Mod_CO1_F01: 5'-GGAGCAGGTACCGGATGAAC-3'
- Aus_Mod_CO1 _R01: 5'-AGAGATCTACTGACGCTCCTGA-3'
- Aus_Mod_CO1 _P01: 5'-FAM-ACCCTCCCCTGTCTAGAAATATTGCTCAC-BHQ-1-3'

Table S38: *Species-specific primer/probe assay for* Elminius modestus *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Austrominius modestus	CO1	76 bp			
Aus_Mod_CO1_F01	GGAGCAGGTACCGGATGAAC		60.2	20	60
Aus_Mod_CO1 _R01	AGAGATCTACTGACGCTCCTGA		59.8	22	50
Aus_Mod_CO1 _P01	ACCCTCCCCTGTCTAGAAATATTGCTCAC		65.6	29	48

Related species	**F01**	**R01**	**P01**
Epopella plicata	3	6	6
Austrobalanus imperator	3	2	6

Species 39: Ficopomatus enigmaticus

Full name: *Ficopomatus enigmaticus* (Fauvel, 1923)

The genus *Ficopomatus* comprises five species (Ten Hove & Kupriyanova 2009), and three of these (*Ficopomatus macrodon, F. miamiensis* and *F. enigmaticus*) are represented with nuclear 18s and 28s nucleotide sequence information on the NCBI database.

For detection of eDNA from 28s nuclear DNA from Ficopomatus enigmaticus two sets of primers and probes are recommended (Table S39.1):

- Fic_eni_28S_F01: 5'-GCAAGGTAGCTTGTCGTCCT-3',
- Fic_eni_28S_R01: 5'-GCGCACCAGACAGTCAGTAA-3',
- Fic_eni_28S_P01: 5'-FAM-CGGCGCAAGAACTTATAGGTTGGCTAT-BHQ-1-3'

Table S39.1: *Species specific primer/probe assay for* Ficopomatus enigmaticus *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Ficopomatus enigmaticus	28S	72 bp			
Fic_eni_28S_F01	GCAAGGTAGCTTGTCGTCCT		59.5	20	55
Fic_eni_28S_R01	GCGCACCAGACAGTCAGTAA		60.1	20	55
Fic_eni_28S_P01	CGGCGCAAGAACTTATAGGTTGGCTAT		68.6	27	48

Related species	**Forward**	**Reverse**	**Probe**
Amplicaria spiculosa	8	10	11
Apomatus globifer	10	12	13
Bushiella abnormis	9	11	9
Circeis armoricana	5	13	11
Circeis spirillum	4	13	10
Crucigera inconstans	10	12	13
Crucigera tricornis	9	13	14
Crucigera zygophora	10	12	12
Ditrupa arietina	5	5	13
Eulaeospira convexis	8	9	12
Ficopomatus enigmaticus	0	0	0
Ficopomatus macrodon	4	12	11
Ficopomatus miamiensis	1	3	6
Filograna implexa	8	9	10
Filogranella elatensis	9	10	12
Filogranula stellata	9	11	10
Floriprotis sabiuraensis	10	11	13
Galeolaria hystrix	7	6	12
Hydroides brachyacanthus	10	13	12
Hydroides elegans	8	11	12
Hydroides ezoensis	9	11	12
Hydroides minax	9	11	12
Hydroides pseudouncinatus	10	11	12
Hydroides sanctaecrucis	9	12	11
Hydroides trivesiculosus	9	12	12
Janua pagenstecheri	11	7	9
Josephella marenzelleri	11	12	13
Jugaria quadrangularis	11	12	10
Laminatubus alvini	5	7	9

Marifugia cavatica	1	3	5
Metalaeospira tenuis	8	11	11
Neodexiospira brasiliensis	7	9	11
Neodexiospira nipponica	6	9	9
Neodexiospira steueri	9	10	10
Neovermilia globula	8	3	14
Paradexiospira vitrea	5	14	8
Paraprotis dendrova	7	10	10
Pileolaria marginata	12	12	11
Pileolaria militaris	15	12	15
Placostegus tridentatus	5	11	8
Pomatoceros lamarckii	7	7	9
Pomatostegus stellatus	7	9	15
Protolaeospira eximia	12	10	11
Pseudovermilia occidentalis	10	9	10
Rhodopsis pusilla	6	11	13
Semivermilia elliptica	8	11	10
Serpula columbiana	10	12	12
Serpula uschakovi	10	12	12
Serpula vermicularis	9	11	15
Serpula vittata	12	11	12
Serpula watsoni	9	11	13
Simplaria potswaldi	12	12	11
Spirobranchus corniculatus	4	9	7
Spirobranchus lima	8	10	9
Spirorbis bifurcatus	10	11	8
Spirorbis bushi	10	13	9
Spirorbis corallinae	10	12	10
Spirorbis rupestris	11	12	10
Spirorbis spirorbis	11	13	10
Spirorbis tridentatus	11	12	8
Vermiliopsis pygidialis	8	10	12
Vinearia koehleri	12	11	11

In addition a second set (Table S39.2) is also recommended to enable detection of eDNA from 18S nuclear DNA from Ficopomatus enigmaticus:
Fic_eni_18S_F02: 5'-TTGACGGCGCTTACACATAG-3',
Fic_eni_18S_R02: 5'-ATAAGCCACCCACGGTCAC-3',
Fic_eni_18S_P02: 5'-FAM-CTCATGATTGTCGGCATCTGGCAAC-BHQ-1-3'

Table S39.2 *Species specific primer/probe assay for* Ficopomatus enigmaticus *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Ficopomatus enigmaticus	18S	103 bp			
Fic_eni_18S_F02	TTGACGGCGCTTACACATAG		59.9	20	50
Fic_eni_18S_R02	ATAAGCCACCCACGGTCAC		60.8	19	58
Fic_eni_18S_P02	CTCATGATTGTCGGCATCTGGCAAC		70.7	25	52

Related species	**Forward**	**Reverse**	**Probe**
Amphicorina mobilis	14	8	1
Amplicaria spiculosa	14	12	13
Apomatus globifer	15	9	12
Apomatus voightae	11	8	12

Bathyvermilia eliasoni	12	5	11
Bispira melanostigma	14	8	1
Bushiella abnormis	11	9	24
Calcisabella piloseta	15	7	1
Chitinopoma serrula	12	4	11
Circeis armoricana	14	9	31
Circeis spirillum	15	8	25
Crucigera inconstans	11	5	1
Crucigera tricornis	12	5	1
Crucigera zygophora	13	8	1
Ditrupa arietina	5	3	15
Echinofabricia alata	12	16	17
Eulaeospira convexis	13	11	2
Ficopomatus enigmaticus	0	0	0
Ficopomatus enigmaticus	0	0	0
Ficopomatus macrodon	3	7	13
Ficopomatus miamiensis	3	1	6
Ficopomatus shenzhensis	2	2	11
Filograna implexa	13	7	19
Filogranella elatensis	13	8	12
Filogranula stellata	12	4	11
Floriprotis sabiuraensis	11	5	1
Galeolaria caespitosa	6	4	15
Galeolaria hystrix	6	5	14
Gunnarea capensis	12	7	0
Hyalopomatus biformis	5	4	13
Hyalopomatus mironovi	5	3	14
Hydroides brachyacanthus	11	5	1
Hydroides elegans	12	9	2
Hydroides ezoensis	12	6	1
Hydroides minax	11	5	1
Hydroides norvegicus	13	3	2
Hydroides novaepommeraniae	11	5	1
Hydroides pseudouncinatus	12	6	0
Hydroides sanctaecrucis	11	5	1
Hydroides trivesiculosus	11	5	1
Hydroides tuberculatus	11	5	1
Janua pagenstecheri	12	12	14
Josephella marenzelleri	12	4	14
Jugaria quadrangularis	11	9	24
Laminatubus alvini	5	4	13
Manayunkia athalassia	15	13	22
Marifugia cavatica	1	2	10
Metalaeospira tenuis	13	11	2
Metavermilia acanthophora	11	7	14
Neodexiospira brasiliensis	13	12	21
Neodexiospira nipponica	13	12	21
Neodexiospira steueri	13	12	18
Neovermilia globula	13	5	0
Novafabricia labrus	13	13	19
Paradexiospira vitrea	15	9	29
Paraprotis dendrova	13	9	14
Pileolaria marginata	12	9	21
Pileolaria militaris	13	9	26

Pomatoceros lamarckii	5	3	13
Pomatoceros taeniata	5	3	14
Pomatoceros triqueter	5	3	15
Pomatoleios kraussii	6	3	14
Pomatostegus stellatus	12	4	12
Protis hydrothermica	13	8	17
Protolaeospira capensis	13	11	2
Protolaeospira eximia	12	11	2
Protolaeospira tricostalis	13	11	2
Protula atypha	15	7	13
Protula bispiralis	13	9	12
Protula palliata	13	9	12
Protula tubularia	16	9	12
Pseudochitinopoma occidentalis	7	3	13
Pseudovermilia occidentalis	15	8	24
Rhodopsis pusilla	15	6	23
Romanchella quadricostalis	13	11	2
Sabella spallanzanii	14	7	1
Sabellaria alveolata	12	7	0
Selenidium neosabellariae	9	16	1
Selenidium sensimae	8	15	2
Semivermilia elliptica	15	8	24
Serpula columbiana	11	5	1
Serpula concharum	10	5	1
Serpula jukesii	11	5	1
Serpula uschakovi	12	8	1
Serpula vermicularis	11	5	1
Serpula vittata	11	6	1
Serpula watsoni	11	5	1
Simplaria potswaldi	12	9	24
Spirobranchus cariniferus	5	3	13
Spirobranchus corniculatus	5	4	15
Spirobranchus latiscapus	5	3	15
Spirobranchus lima	5	3	13
Spirorbis bifurcatus	13	9	24
Spirorbis bushi	13	11	25
Spirorbis corallinae	12	9	26
Spirorbis rupestris	12	9	26
Spirorbis spirorbis	12	9	25
Spirorbis tridentatus	12	9	26
Vermiliopsis infundibulum	12	8	20
Vermiliopsis labiata	11	8	21
Vermiliopsis pygidialis	8	9	16
Vermiliopsis striaticeps	12	8	21
Vinearia koehleri	11	8	25

Because there are species of *Ficopomatus* that are not represented by nucleotide sequences on the NCBI database, the recommended primer and probe systems suggested here are may or may not be able to distinguish between *Ficopomatus* enigmaticus and other non-sequenced species of *Ficopomatus*.

Species 40: Marenzelleria viridis

Full name: *Marenzelleria viridis* (Verrill, 1873)

The genus *Marenzelleria* comprises seven species (WoRMS Editorial Board 2015). In European seas two species of *Marenzelleria* are reported (Bellan 2001). On the NCBI GenBank database five species of Marenzelleria are represented by nucleotide sequence information. It might be that there are multiple haplotypes of (Blank & Bastrop 2009) of *Marenzelleria viridis* occurring in North European seas, and it is thus difficult to assure the recommended primer-probe system will be able to detect all haplotypes of *Marenzelleria viridis* occurring in Northern European seas, while also not returning false positives from other co-occuring species of *Marenzelleria* (Blank & Bastrop 2009, Bellan 2001). To accommodate this multiple primer-probe systems are here recommended for being able to discriminate between the various species and haplotypes of *Marenzelleria.*

For the mirochondrial 16s gene region the following primers and probes are recommended for detection of eDNA from Marenzelleria viridis (Table S40.1):

- Mar_vir_16S_F01: 5'-AAGCTCCTATAGCTTACGCAAA-3',
- Mar_vir_16S_R01: 5'-GAAGATTTAGGTCTTCTCTTGTCG-3',
- Mar_vir_16S_P01: 5'-FAM-GAACTTTACCTACAGAAATTTTGTTGGGG-BHQ1-3'

Table S40.1: *Species specific primer/probe assay for* Marenzelleria viridis *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Marenzelleria viridis	16S	76 bp			
Mar_vir_16S_F01	AAGCTCCTATAGCTTACGCAAA		57.6	22	41
Mar_vir_16S_R01	GAAGATTTAGGTCTTCTCTTGTCG		57.8	24	41
Mar_vir_16S_P01	GAACTTTACCTACAGAAATTTTGTTGGGG		64.8	29	38

Related species	**Forward**	**Reverse**	**Probe**
Laonice norgensis	16	12	14
Malacoceros fuliginosus	15	2	10
Malacoceros indicus	16	13	13
Malacoceros indicus	16	13	13
Marenzelleria arctia	5	0	7
Marenzelleria bastropi	4	0	3
Marenzelleria neglecta	2	0	2
Marenzelleria viridis	0	0	0
Marenzelleria wireni	4	0	3
Rhynchospio foliosa	15	5	11
Scolelepis eltaninae	17	3	14

In case the recommended system for detection of mtDNA-16s from *M. viridis* turns out to be unspecific, a system for the mitochondrial cytochrome b gene region is also recommended for detection of eDNA from *Marenzelleria viridis* (Table S40.2):

- Mar_vir_Cytb_F02: 5'-CGATTCTTTGCGTTCCATTT-3'
- Mar_vir_Cytb_R02: 5'-GCCAAGAGGGTTATTTGATCC-3'
- Mar_vir_Cytb_P02: 5'-FAM-CCCTCTCAGGGGTTCACCTTCTATTTC-BHQ-1-3'

Table S40.2: *Species specific primer/probe assay for* Marenzelleria viridis *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	Gene	Product size	Temp	Length	GC
Marenzelleria viridis	Cytb	102 bp			
Mar_vir_Cytb_F02	CGATTCTTTGCGTTCCATTT		60.1	20	40
Mar_vir_Cytb_R02	GCCAAGAGGGTTATTTGATCC		59.8	21	48
Mar_vir_Cytb_P02	CCCTCTCAGGGGTTCACCTTCTATTTC		67.9	27	52

Related species	Forward	Reverse	Probe
Boccardia proboscidea	6	4	12
Boccardiella hamata	4	6	14
Malacoceros fuliginosus	2	6	8
Marenzelleria arctia	4	4	9
Marenzelleria bastropi	5	3	8
Marenzelleria neglecta	1	6	5
Marenzelleria viridis	0	0	0
Marenzelleria wireni	2	5	6
Polydora brevipalpa	5	4	14
Polydora hoplura	5	5	14
Polydora websteri	5	2	12

Species 41: Ocenebra inornata

Full name: *Ocenebra inornata* Récluz, 1851

The *Ocenebra* genus comprises seven marine species. The genus belongs to the subfamily Ocenebrinae which has 28 genera, according to WoRMS.

Out of the seven marine species, sequence data was only available for *O. inornata* and *O. erinacea*. The sequence data from *O. inornata* was compared to related species and to species showing sequence similarity via sequence BLAST analysis. Two assays are designed, spanning two different regions of the CO1 segment.

The first set of primers and probe to target *O. inornata* is:

- Oce_Ino_CO1_F01: 5'-GGCTTGTCGGAACTGCCTTA-3'
- Oce_Ino_CO1_R01: 5'-TCATCCCCAAGCAAAGCTCC-3'
- Oce_Ino_CO1_P01: 5'-FAM-AGTCTTCTTATTCGAGCTGAACTAGGGCA-BHQ-1-3'

Table S41.1: *Species-specific primer/probe assay for* Ocenebra inornata *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	Gene	Product size	Temp	Length	GC
Ocenebra inornata	CO1	73 bp			
Oce_Ino_CO1_F01	GGCTTGTCGGAACTGCCTTA		60.3	20	55
Oce_Ino_CO1_R01	TCATCCCCAAGCAAAGCTCC		60.3	20	55
Oce_Ino_CO1_P01	AGTCTTCTTATTCGAGCTGAACTAGGGCA		65.3	29	45

Related species	F01	R01	P01
Ocenebra erinacea	4	7	2
Ocinebrina edwardsii	4	7	1
Ocinebrina lurida	1	2	1
Purpura persica	7	6	0
Tiariturris spectabilis	7	5	0
Turris hidalgoi	4	1	0
Xenuroturris albino	4	3	1

The second set of primers and probe designed to target *O. inornata*:

- Oce_Ino_CO1_F02: 5'-ACCCCCTGCCTTACTTCTTTT-3'
- Oce_Ino_CO1_R02: 5'-ACAGATCCTCCAGCATGAGC-3'
- Oce_Ino_CO1_P02: 5'-FAM-TGGACTGTCTACCCTCCATTAGCTGGT-BHQ-1-3'

Table S41.2: *Species-specific primer/probe assay for* Ocenebra inornata *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Ocenebra inornata	CO1	114 bp			
Oce_Ino_CO1_F02	ACCCCCTGCCTTACTTCTTTT		59.2	21	48
Oce_Ino_CO1_R02	ACAGATCCTCCAGCATGAGC		59.5	20	55
Oce_Ino_CO1_P02	TGGACTGTCTACCCTCCATTAGCTGGT		66.4	27	52

Related species	**F01**	**R01**	**P01**
Ocenebra erinacea	5	1	7
Ocinebrina edwardsii	5	1	5
Ocinebrina lurida	3	1	3
Purpura persica	8	3	4
Tiariturris spectabilis	7	2	6
Turris hidalgoi	5	3	5
Xenuroturris albino	10	5	4

Species 42: Potamopyrgus antipodarum

Full name: *Potamopyrgus antipodarum* Gray, 1843.

The genus *Potamopyrgus* consists of 11 marine species and belongs to the family Tateidae (WoRMS 2015).

An assay for *P. antipodarum* has already been published and validated based on the cytochrome b region (Goldberg *et al.* 2013). However, sequence data on the cytochrome b region is only available for one sister-species at NCBI; hence occurrence of non-target sister species may lead to false positives.

- Pot_Ant_Cytb_F01: 5'- TGTTTCAAGTGTGCTGGTTTAYA-3'
- Pot_Ant_Cytb_R01: 5'-CAAATGGRGCTAGTTGATTCTTT-3'
- Pot_Ant_Cytb_P01: 5'-FAM-CCTCGACCAATATGTAAAT-MGB-NFQ-3'

The annealing temperature was 60 °C in the study by Goldberg *et al.* (2013), whereas the values in Table S42 are the optimal temperatures calculated in Geneious version 9.0.4.

Table S42: *Species-specific primer/probe assay for* Potamopyrgus antipodarum *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Potamopyrgus antipodarum	Cytb	93 bp			
Pot_Ant_Cytb_F01	TGTTTCAAGTGTGCTGGTTTAYA		56.6 - 59.2	23	35 - 39
Pot_Ant_Cytb_R01	CAAATGGRGCTAGTTGATTCTTT		55.2 - 57.3	23	35 - 39
Pot_Ant_Cytb_P01	CCTCGACCAATATGTAAAT*		49.5	19	36
*TaqMan MGB probe					

Related species	**F01**	**R01**	**P01**
Potamopyrgus estuarinus	1	1	0

Species 43: Pseudochattonella verruculosa

Full name: *Pseudochattonella verruculosa* (Y.Hara & M.Chihara) S.Tanabe-Hosoi, D.Honda, S.Fukaya, Y.Inagaki & Y.Sako

The genus *Pseudochattonella* only contains the two species *Pseudochattonella farcimen* and *Pseudochattonella verruculosa*. The two species are more or less impossible to morphologically tell apart after fixation in lygol. Due to this a molecular method has been developed and to date serve as the best solution to tell apart the two species (Dittami *et al.* 2013). However, the designed primers (best possible) only have two mismatches each, respectively. According to the published qPCR protocol it is relatively difficult even with the molecular method to determine if the amplification in the qPCR reaction is caused by the presence of the one or the other species (Dittami *et al.* 2013):

- PverF: 5'-GGTCCCGGGGAGAAGTC-3'
- PverR: 5'-AAGCAACTCGACTCCATTAGC-3'

No other mismatches exist within the sequence between the two primers. Therefore, no probe was designed. However, it has come to our attention that a primer/probe assay is under development and awaiting publication (Eckford-Soper & Daugbjerg (in press)). With this new assay it is expected that differentiation between the two *Pseudochattonella* species will possible.

Table S43: *Species specific primer/probe assay for* Pseudochattonella verruculosa *with, target gene, product size, melting temperature, primer/probe length, and GC ratio (%)*

Species	Gene	Product size	Temp	Length	GC
Pseudochattonella verruculosa	28S	91 bp			
PverF	GGTCCCGGGGAGAAGTC		59.8	17	71
PverR	AAGCAACTCGACTCCATTAGC		59.5	21	48

Species 44: Codium fragile* subsp*. fragile

Full name: *Codium fragile* subsp. *fragile* (Suringar) Hariot, 1889

Codium fragile subsp. *fragile* is as the name indicates a subspecies to *Codium fragile* together with three other subspecies. These subspecies belongs to the genus *Codium* showing a very large number of species (*n*137). Within the subspecies group only the sequence for the ribosomal protein S3 (*rps*3) and L16 (*rpl*16) is represented by all four subspecies:

- Cod_sub_fra_rps3_F01: 5'-AAATGTTACTCATGCTCCAACC-3'
- Cod_sub_fra_rps3_R01: 5'-CCAATAGGGCTGTAATGCC-3'
- Cod_sub_fra_rps3_P01: 5'-FAM-AAGTAAATTTCGTAAAAATCATCGCGG-BHQ-1-3'

However, the sequence showed no usable mismatches among the four subspecies (represented by *Codium fragile* subsp. *novae-zelandiae* in table S44). Despite this and the relative large size of the amplicon the rps3 sequence provides a good basis for future eDNA work on the species. The assay was designed based on the alignment of sequence information from no less than 71 species within the genus *Codium*. However, due to simplicity only nine species with less than six mismatches have been listed in the table S44. Five species (first five in the table) were selected to represent the remaining 62 species from the comparison. These are also listed in table S44, however without mismatch information.

Table S44: *Species specific primer/probe assay for* Codium fragile *subsp.* fragile *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species. Problematic species are marked in bold*

Species	Gene	Product size	Temp	Length	GC
Codium fragile subsp. *fragile*	*rps*3-*rpl*16	138 bp			
Cod_sub_fra_rps3_F01	AAATGTTACTCATGCTCCAACC		58.4	22	41
Cod_sub_fra_rps3_R01	CCAATAGGGCTGTAATGCC		57.5	19	53
Cod_sub_fra_rps3_P01	AAGTAAATTTCGTAAAAATCATCGCGG		61.1	27	33

Related species	F01	R01	P01
Codium fragile* subsp. *novae-zelandiae	**0**	**0**	**0**
Codium adhaerens	7	6	6
Codium arabicum	6	6	6
Codium barbatum	8	5	4
Codium bursa	8	6	2
Codium capitatum	7	5	3
Codium capitulatum	6	7	7
Codium cranwelliae	4	6	3
Codium cylindricum	3	4	3
Codium decorticatum	2	4	3
Codium duthieae	2	4	2
Codium galeatum	3	3	2
Codium minus	4	8	3
Codium papenfussii	3	4	2
Codium sp. DML66031	1	5	3
Codium yezoense	**1**	**2**	**1**
Codium cf. bursa DHO2-176			
Codium cf. dimorphum C29			
Codium cf. dimorphum C77			
Codium cf. flabellatum DHO-009			
Codium cf. fragile AU5			

Codium cf. latum ASH-018
Codium cf. latum C51
Codium cf. minus DHO-015
Codium cf. minus DHO2-188
Codium cf. tenue HV608
Codium contractum
Codium convolutum
Codium coralloides
Codium dimorphum
Codium effusum
Codium geppiorum
Codium gracile
Codium hubbsii
Codium hubbsii
Codium intertextum
Codium intricatum
Codium isthmocladum
Codium isthmocladum subsp. clavatum
Codium laminarioides
Codium latum
Codium lucasii
Codium lucasii subsp. capense
Codium megalophysum
Codium muelleri
Codium ovale
Codium parvulum
Codium platylobium
Codium prostratum
Codium repens
Codium saccatum
Codium setchellii
Codium sp. 'arenicola'
Codium sp. DB2006
Codium sp. DHO-007
Codium sp. DHO2-196
Codium sp. DHO2-348
Codium sp. DML30929
Codium sp. DML30930
Codium sp. DML40218
Codium sp. DML40227
Codium sp. DML65827
Codium sp. DML65829
Codium sp. HV1061
Codium sp. HV1068
Codium spinescens
Codium spongiosum
Codium subtubulosum
Codium taylorii
Codium vermilara

Species 45: Carassius auratus

Full name: *Carassius auratus* (Linnaeus, 1758).

The genus *Carassius* belongs to the family Cyprinidae and includes five species (Froese & Pauly 2015). Occurrences of hybridization between *C. auratus* and other closely related species, including *Cyprinus carpio,* are reported. Since the mitochondrial genome of these hybrids is maternally inherited the assay will be unable to distinguish hybrids from normal individuals in cases where the mitochondrion genome originates from *C. auratus* (Yan *et al.* 2005).

An assay for *C. auratus* based on the cytochrome b sequence has been published and validated (Nathan *et al.* 2014). That assay consists of a primer pair with the sequences:

- Car_Aur_Cytb_F01: 5'-GCTTCTCCGTAGATAATG-3'
- Car_Aur _Cytb_R01: 5'-TTCGTGAAGAAACAGTAG-3'

Testing the primers reveals limited variation between *C. gibelio* and *C. auratus*. To increase specificity a probe was tested within the amplicon, but the amplicon had no optimal binding sites. Furthermore, the most common intraspecies nucleotide polymorphisms are not represented within the primers sequence and will therefore potentially not bind to the targeted organisms DNA.

Table S45.1: *Species-specific primer/probe assay for* Carassius auratus *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	Gene	Product size	Temp	Length	GC
Carassius auratus auratus	Cytb	106 bp			
Car_Aur_Cytb_F01	GCTTCTCCGTAGATAATG		49.3	18	44
Car_Aur_Cytb_R01	TTCGTGAAGAAACAGTAG		49.2	18	39

Related species	F01	R01	P01
C. auratus	1	1	-
C. gibelio	1	1	-
C. carassius	2	2	-
Cyprinus carpio	2	2	-
C. auratus x Megalobrama amblycephala EU518483	1	1	-
C. auratus x Megalobrama amblycephala EU518484	1	1	-
C. auratus x 2 Megalobrama amblycephala EU518485	1	2	-
C. auratus x Cyprinus carpio NC_006136	1	1	-
C. auratus x Cyprinus carpio AY694420	1	1	-
C. auratus x Cyprinus carpio GU186888	1	1	-
C. auratus x Cyprinus carpio x Carassius cuvieri AY771781	1	2	-
C. auratus x Cyprinus carpio x Carassius cuvieri C_006387	1	2	-
Cyprinus carpio x C. auratus NC_018038	2	1	-
Cyprinus carpio x C. auratus NC_026543	3	1	-

A secondary assay was designed with the inclusion of a probe with following sequences:

- Car_Aur_Cytb_F02: 5'-GACAGCCTTTGTCGGTTATGT-3'
- Car_Aur_Cytb_R02: 5'-TATGGCACGGCGGATAGAAG-3'
- Car_Aur_Cytb_P02: 5'-TCCTTTTGAGGCGCTACAGTAATCACAAAC-BHQ-1-3'

A comparison between *C. auratus* and *C. gibelio* showed limited variation, which confines the ability to distinguish these two species. This is potentially due to taxonomic confusion between *C. auratus auratus* and *C. gibelio* and their complex modes of reproduction (Froese & Pauly 2015)

Table S45.2: *Species-specific primer/probe assay for* Carassius auratus *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Carassius auratus auratus	Cytb	90 bp			
Car_Aur_Cytb_F02	GACAGCCTTTGTCGGTTATGT		58.6	21	47
Car_Aur_Cytb_R02	TATGGCACGGCGGATAGAAG		59.7	20	55
Car_Aur_Cytb_P02	TCCTTTTGAGGCGCTACAGTAATCACAAAC		65.7	30	43

Related species	**F01**	**R01**	**P01**
C. auratus	0	0	0
C. gibelio	0	1	0
C. carassius	2	3	1
Cyprinus carpio	4	3	1
C. auratus x Megalobrama amblycephala EU518483	0	1	1
C. auratus x Megalobrama amblycephala EU518484	0	0	0
C. auratus x 2 Megalobrama amblycephala EU518485	0	3	0
C. auratus x Cyprinus carpio NC_006136	0	0	0
C. auratus x Cyprinus carpio AY694420	0	0	0
C. auratus x Cyprinus carpio GU186888	0	1	0
C. auratus x Cyprinus carpio x Carassius cuvieri AY771781	0	3	1
C. auratus x Cyprinus carpio x Carassius cuvieri NC_006387	0	3	1
Cyprinus carpio x C. auratus NC_018038	4	3	1
Cyprinus carpio x C. auratus NC_026543	4	3	4

Species 46: Cyprinus carpio

Full name: *Cyprinus carpio* Linnaeus, 1758.

The genus Cyprinus comprises two species that can occur in brachish waters and 24 species in total. The genus belongs to the family Cyprinidae containing 3032 species (Froese & Pauly 2015; WoRMS 2015).

Hybridization between *C. carpio* and *Carassius auratus,* is reported. The mitochondrial genome of these hybrids is maternally inherited and the assay will hence not be able to distinguish hybrids from normal individuals in cases where the mitochondrion genome originates from *C. carpio* (Yan *et al.* 2005).

Following primer pair for *C. carpio* has already been published based on the D-loop region (Turner *et al.* 2014):

- Cyp_Car_dloop_F01: 5'-GAGTGCAGGCTCAAATGTTAAA-3'
- Cyp_Car_dloop_R01: 5'-GTAAGGATAAGTTGAACTAGAGACAG-3'

A BLAST analysis and local alignment show that the primers are specific to the species within the genus *Cyprinus*. As *C. carpio* is the only species occurring in Danish territorial waters it is therefore expected that the assay is specific (Froese & Pauly 2015).

An internal probe within the amplicon was developed to increase the specificity of the assay. It has the following sequence:

- Cyp_Car_dloop_P01: 5'-FAM-AAGGTTGAACATTTTCCTTGTATGTGAT-BHQ-1-3'

Table S46: *Species-specific primer/probe assay for* Cyprinus carpio *with target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	Gene	Product size	Temp	Length	GC
Cyprinus carpio	D-loop	146 bp			
Cyp_Car_dloop_F01	GAGTGCAGGCTCAAATGTTAAA		57.5	22	41
Cyp_Car_dloop_R01	GTAAGGATAAGTTGAACTAGAGACAG		56.9	26	39
Cyp_Car_dloop_P01	AAGGTTGAACATTTTCCTTGTATGTGAT		60.2	28	32

Related species	F01	R01	P01
Cyprinus acutidorsalis	0	0	0
Cyprinus rubrofuscus	0	0	0
Cyprinus pellegrini	0	0	2
Cyprinus multitaeniata	0	0	1
Cyprinus melanes	0	0	0
Carassius gibelio	4	6	1
Carassius cuvieri	4	6	3
Carassius auratus auratus	5	6	1
Carassius carassius	10	3	2
C. auratus auratus × *C. carpio*	4	7	1
C. auratus auratus × *C. carpio* × *C. cuvieri*	4	6	1

Species 47: Mya arenaria

Full name: *Mya arenaria* Linnaeus, 1758

The genus *Mya* comprises four species and the genus belongs to the family Myoidea. Within the family the CO1 gene is good represented and therefore selected for the assay design:

- Mya_are_CO1_F01: 5'-CGTGCAGGATGTCACCCG-3'
- Mya_are_CO1_R01: 5'-CGCCAAAACTGGCATTGCTAA-3'
- Mya_are_CO1_P01: 5'-FAM-CTGTAGGTCTATTGGAGTTACCAGGTTT-HBQ-1-3'

Table S47: *Species specific primer/probe assay for* Mya arenaria *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Mya arenaria	CO1	95 bp			
Mya_are_CO1_F01	CGTGCAGGATGTCACCCG		60.8	18	67
Mya_are_CO1_R01	CGCCAAAACTGGCATTGCTAA		59.5	21	48
Mya_are_CO1_P01	CTGTAGGTCTATTGGAGTTACCAGGTTT		67.2	28	43
Related species*	**Forward**	**Reverse**	**Probe**		
Mya truncata	2	7	5		
Varicorbula dissimilis	7	5	8		
Corbula amurensis	7	5	6		
Corbula erythrodon	4	5	3		
Corbula gibba	9	5	12		
Corbula tunicata	5	5	8		
Potamocorbula fasciata	9	5	6		

* not necessarily related but show strong sequence similarity to target species

Species 48: Penilia avirostris

Full name: *Penilia avirostris* Dana, 1849

Penilia avirostris is the only species in the genus *Penilia*. However, in the Family Sididae comprise a fairly larger number of species in close relationship to *Penilia avirostris*. For a large portion of Sididae the gene sequence for CO1 was available and was found to be very divergent between the species (Table S48):

- Pen_avi_CO1_F01: 5'-AGCGGGGATTTCATCAATCC-3'
- Pen_avi_CO1_R01: 5'-CCACACAAAGAGGGGAATAC-3'
- Pen_avi_CO1_P01: 5'-FAM- CGACAATTGTAAATATACGATCTAAAGGA-BHQ-1-3'

Table S48: *Species specific primer/probe assay for* Penilia avirostris *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species*

Species	**Gene**	**Product size**		**Temp**	**Length**	**GC**
Penilia avirostris	CO1	103 bp				
Pen_avi_CO1_F01	AGCGGGGATTTCATCAATCC			58.4	20	50
Pen_avi_CO1_R01	CCACACAAAGAGGGGAATAC			58.4	20	50
Pen_avi_CO1_P01	CGACAATTGTAAATATACGATCTAAAGGA			63.3	29	31
Related species*	**Forward**	**Reverse**	**Probe**			
Sarsilatona serricauda	6	5	10			
Sarsilatona cf. serricauda	3	6	7			
Sida crystallina americana	5	8	8			
Latonopsis australis	6	4	6			
Latonopsis cf. australis	6	6	6			
Diaphanosoma birgei	4	5	8			
Diaphanosoma brachyurum	6	6	11			
Diaphanosoma brevireme	6	6	4			
Diaphanosoma cf. amurensis	6	7	8			
Diaphanosoma cf. dubium	7	9	9			
Diaphanosoma cf. heberti	6	6	10			
Diaphanosoma cf. macrophthalma	6	4	9			
Diaphanosoma cf. orientalis	5	7	6			
Diaphanosoma dubium	6	8	11			
Diaphanosoma sp. 1 MEG-2008	5	9	5			
Diaphanosoma sp. 1 MEG-2013	5	9	5			
Diaphanosoma sp. HE-091	5	4	6			
Diaphanosoma sp. HE-099	5	4	6			
Diaphanosoma sp. HE-572	6	6	7			
Diaphanosoma sp. HE-572.1	6	6	7			
Diaphanosoma sp. HE-648.1	6	4	8			
Diaphanosoma sp. HE-649	6	4	8			
Diaphanosoma sp. HE-649.1	6	4	8			
Diaphanosoma sp. HE-652	6	4	8			
Diaphanosoma sp. IW-2015-1	5	4	6			
Diaphanosoma sp. JRdW-2005	6	6	11			
Diaphanosoma spinulosum	6	5	6			

* not necessarily related but show strong sequence similarity to target species

Species 49: Diadumene lineata

Full name: *Diadumene lineata* (Verrill, 1870)

The genus *Diadumene* comprises 11 species (WoRMS Editorial Board 2015). From the family Diadumenidae only *Diadumene cineta* Stephenson, 1925 and *Haliplanella luciae* (Verrill, 1899) appear to be recorded in European seas (van der Land & den Hartog, 2001). Unfortunately, very few species of *Diadumene* are represented with nucleotide sequence information on the NCBI GenBank database (Nov-2015). Also, very few species of both Diadumenidae and the suborder Nynantheae are represented by nucleotide sequence data on NCBI GenBank. Only *Diadumene cincta* and *Diadumene leucolena* have nucleotide sequence information deposited on the NCBI GenBank database. Without any sequence information available for *Diadumene lineata* it is currently not possible to develop a species-specific primer-probe system that only detects *Diadumene lineata* and not any other species of *Diadumene*. The primer-probe systems recommended here are therefore based on various other representatives from Nynantheae.

As only *Diadumene cincta* and *Diadumene leucolena* have nucleotide sequence information deposited on the NCBI GenBank database, the recommended primers and probes have been designed to detect eDNA from these two species instead. More universal primers that will detect eDNA from *Diadumene* spp. are currently not possible to design with the sequence information deposited on the NCBI GenBank database.

A comparison of fragments of nuclear 18s gene markers from Nynantheae unfortunately returns *Diadumene* as a polyphyletic genus, which makes a more universal primer-probe system unable to differentiate between *Diadumene* and various other genera in Nynantheae. The nuclear 18S gene fragments deposited at NCBI GenBank have the highest nucleotide diversity among different gene marker fragments deposited on NCBI, but even the most variable region of 18s from Nynantheae does not have sufficient variation to distinguish between *Diadumene* and several other genera within Nynantheae. This excludes the possibility of designing primers and probes specific for only *Diadumene* spp.

Here two primer-probe systems are recommended, that are targeting eDNA from *Diadumene cincta* and *Diadumene leucolena*. However, it is not possible to determine whether these two systems will enable detection of eDNA from any of the other nine species of *Diadumene*, or if these two systems will be able to differentiate between *Diadumene cincta* and *Diadumene leucolena* and any other species of *Diadumene* spp. Also, the limited nucleotide variation in the 18S gene fragments introduce a risk of obtaining false positive eDNA detection from other species from Nynantheae, as even the most variable region of 18S is unable to distinguish between all genera and species of Nynantheae in just a short stretch of 70 – 120 bp size DNA fragment.

For detection eDNA-fragments form 18S-nDNA from *Diadumene leucolena* the following primers and probes are recommended (Table S49.1):

- Dia_leu_18S_F01: 5'-ACTGACCGGGCTGTTCTTCT-3',
- Dia_leu_18S_R01: 5'-GTCACAAGTCCTACCGCACA-3',
- Dia_leu_18S_P01: 5'-FAM-AAGGACTGCGTGTGCTCTTAGCTGAG-BHQ1-3',

Table S49.1: *Species specific primer/probe assay for* Diadumene leucolena *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	Gene	Product size	Temp	Length	GC
Diadumene leucolena	18S	70 bp			
Dia_leu_18S_F01	CGATTCTTTGCGTTCCATTT		61.2	20	55
Dia_leu_18S_R01	GCCAAGAGGGTTATTTGATCC		59.8	20	55
Dia_leu_18S_P01	CCCTCTCAGGGGTTCACCTTCTATTTC		68.2	26	54

Related species	Forward	Reverse	Probe
Actinauge richardi	1	1	2
Actinia equina	4	3	3
Actinia fragacea	4	4	3
Actinia tenebrosa	4	3	2
Actinostola crassicornis	2	3	3
Adamsia palliata	1	4	3
Alicia mirabilis	0	1	1
Alicia sansibarensis	0	1	1
Allantactis parasitica	1	1	1
Anemonia sulcata	3	6	4
Anemonia viridis	2	6	3
Antholoba achates	4	5	9
Anthopleura artemisia	3	3	3
Anthopleura atodai	4	4	3
Anthopleura elegantissima	6	8	5
Anthopleura handi	5	5	3
Anthopleura krebsi	3	2	2
Anthopleura kurogane	4	5	3
Anthopleura midori	4	3	2
Anthopleura rosea	6	7	4
Anthosactis janmayeni	2	5	3
Aulactinia incubans	3	4	5
Aulactinia stella	3	4	5
Aulactinia vancouverensis	5	4	5
Aulactinia veratra	6	7	4
Aulactinia verrucosa	3	2	2
Bunodactis reynaudi	3	2	2
Bunodosoma grandis	3	4	3
Calliactis japonica	1	1	1
Calliactis parasitica	3	5	3
Calliactis polypus	1	2	1
Calliactis tricolor	1	2	1
Charisea saxicola	0	1	1
Cricophorus nutrix	3	4	4
Cyananthea hourdezi	0	4	1
Diadumene cincta	2	4	5
Diadumene leucolena	0	0	0
Diadumene leucolena	0	0	0
Diadumene sp.	0	1	2
Epiactis australiensis	10	4	8
Epiactis georgiana	3	2	3
Epiactis handi	3	2	3
Epiactis japonica	3	2	3
Epiactis lisbethae	3	2	3
Epiactis prolifera	3	2	3
Epiactis ritteri	3	2	3
Galatheanthemum profundale	1	1	1
Gyractis sesere	4	4	2
Haliplanella lineata	4	10	10
Haliplanella lucia	4	7	7
Hormathia armata	3	5	4
Hormathia lacunifera	3	5	4
Hormathia pectinata	1	1	2

Hormosoma scotti	1	5	3
Isactinia olivacea	6	7	4
Kadosactis antarctica	0	4	1
Metridium senile	0	1	1
Nemanthus nitidus	2	3	2
Ostiactis pearseae	0	2	1
Oulactis muscosa	5	2	2
Paracondylactis hertwigi	4	5	4
Paranthus niveus	0	1	1
Stephanthus antarcticus	3	2	2
Stomphia didemon	4	9	7
Triactis producta	0	2	1
Urticina coriacea	3	4	3
Urticina crassicornis	3	2	4
Urticina fecunda	3	2	2
Urticina grebelnyi	7	2	3

An additional system for detection of *Diadumene cincta* is also recommended, in case *Diadumene lineata* have too distant a molecular evolutionary relationship with *Diadumene leucolena* (Table S49.2):

- Dia_cin_18S_F02: 5'-ACTGACCGAGCTGTCCTTCT-3',
- Dia_cin_18S_R02: 5'-GTCGCAAGTCCCGCCGCACA-3',
- Dia_cin_18S_P02: 5'-FAM-AAAGACCTCGTGTGCTCTTGACTGAG–BHQ-1-3',

Table S49.2: *Species specific primer/probe assay for* Diadumene cincta *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Species	**Gene**	**Product size**	**Temp**	**Length**	**GC**
Diadumene cincta	18S	70 bp			
Dia_cin_18S_F02	ACTGACCGAGCTGTCCTTCT		61.2	20	55
Dia_cin_18S_R02	GTCGCAAGTCCCGCCGCACA		59.8	20	55
Dia_cin_18S_P02	AAAGACCTCGTGTGCTCTTGACTGAG		68.2	26	54

Related species	**Forward**	**Reverse**	**Probe**
Actinauge richardi	1	5	4
Actinia equina	4	3	4
Actinia fragacea	4	2	4
Actinia tenebrosa	4	3	3
Actinostola crassicornis	2	3	2
Adamsia palliata	3	4	4
Alicia mirabilis	2	5	4
Alicia sansibarensis	2	5	4
Allantactis parasitica	1	5	4
Anemonia sulcata	5	2	1
Anemonia viridis	4	4	2
Antholoba achates	6	1	7
Anthopleura artemisia	5	3	4
Anthopleura atodai	6	2	2
Anthopleura elegantissima	4	5	6
Anthopleura handi	5	3	2
Anthopleura krebsi	5	4	3
Anthopleura kurogane	6	3	2
Anthopleura midori	6	3	3
Anthopleura rosea	5	3	3
Anthosactis janmayeni	4	5	4

Aulactinia incubans	5	3	2
Aulactinia stella	5	3	2
Aulactinia vancouverensis	7	3	2
Aulactinia veratra	5	3	3
Aulactinia verrucosa	3	4	3
Bunodactis reynaudi	5	4	3
Bunodosoma grandis	5	2	4
Calliactis japonica	1	5	4
Calliactis parasitica	3	5	4
Calliactis polypus	1	4	4
Calliactis tricolor	1	4	4
Charisea saxicola	2	5	4
Cricophorus nutrix	3	2	5
Cyananthea hourdezi	2	6	4
Diadumene cincta	0	0	0
Diadumene leucolena	2	4	5
Diadumene leucolena	2	4	5
Diadumene sp.	2	3	5
Epiactis australiensis	12	5	10
Epiactis georgiana	5	4	3
Epiactis handi	5	4	2
Epiactis japonica	5	4	2
Epiactis lisbethae	5	4	2
Epiactis prolifera	5	4	2
Epiactis ritteri	5	4	2
Galatheanthemum profundale	1	5	4
Gyractis sesere	4	2	3
Haliplanella lineata	4	6	6
Haliplanella lucia	4	3	3
Hormathia armata	1	3	3
Hormathia lacunifera	1	3	3
Hormathia pectinata	1	5	4
Hormosoma scotti	3	3	4
Isactinia olivacea	5	3	3
Kadosactis antarctica	2	6	4
Metridium senile	2	5	4
Metridium senile	2	5	4
Nemanthus nitidus	2	7	5
Ostiactis pearseae	2	4	4
Oulactis muscosa	7	4	3
Paracondylactis hertwigi	6	3	3
Paranthus niveus	2	5	4
Stephanthus antarcticus	5	4	3
Stomphia didemon	4	5	4
Triactis producta	2	4	4
Urticina coriacea	5	4	2
Urticina crassicornis	5	4	3
Urticina fecunda	5	4	3
Urticina grebelnyi	9	4	2

Species 50: Bonnemaisonia hamifera

Full name: *Bonnemaisonia hamifera* Hariot 1891

In the genus *Bonnemaisonia* there are only four species. To ensure better assay design sequences from available species within the family *Bonnemaisoniales* were also included. Among the sequenced genes for the four species in the genus *Bonnemaisonia* the *rbc*L gene showed the best potential for assay design (table S50):

- Bon_ham_rbcL_F01: 5'-CAATTACTAGATTACCTGGGCA-3'
- Bon_ham_rbcL_R01: 5'-CTTTTACAAAGTCCCGACCT-3'
- Bon_ham_rbcL_P01: 5'-FAM- GGCTTTAGAGTCTATGGTTATGGCACGA-BHQ-1-3'

The total length of the product was 153 bp. The length may become a problem due to DNA degradation and therefore the possibility to extract fragments of such lengths for amplification.

Table S50: *Species specific primer/probe assay for* Bonnemaisonia hamifera *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species*

Species	Gene	Product size	Temp	Length	GC
Bonnemaisonia hamifera	rbcL	153 bp			
Bon_ham_rbcL_F01	CAATTACTAGATTACCTGGGCA		58.4	22	41
Bon_ham_rbcL_R01	CTTTTACAAAGTCCCGACCT		56.4	20	45
Bon_ham_rbcL_P01	GGCTTTAGAGTCTATGGTTATGGCACGA		68.5	28	46

Related species*	Forward	Reverse	Probe
Bonnemaisonia asparagoides	5	3	6
Bonnemaisonia clavata	5	3	6
Bonnemaisonia geniculata	5	5	5
Asparagopsis armata	2	7	5
Asparagopsis taxiformis	6	6	6
Delisea flaccida	5	5	4
Delisea hypneoides	5	5	4
Delisea japonica	5	4	missing
Delisea pulchra	5	3	4
Ptilonia magellanica	6	2	5
Reticulocaulis mucosissimus	5	5	7

* not necessary related but show strong sequence similarity to target species

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Appendix D: Ranking of species

Nr.	Group	Name	DCE	HELCOM	OSPAR	EU-forord-ning	NST 1	NST 2	KJ 1	KJ 2	PRM	Criteria 1 established NIS	Criteria 2 potential NIS	Criteria 3 invasive species	Criteria 4 ease of deter-mination	Criteria 5 eDNA	Score
1	Fish	*Neogobius melanostomus* (Pallas, 1814)	x	x	x			x	x			10		6	3	4	23
2	Inverte-brate	*Crassostrea gigas* (Thunberg, 1793)	x	x	x	x	x		x			10		6	3	3	22
3	Inverte-brate	*Crepidula fornicata* (Linnaeus, 1758)	x	x	x	x		x	x			10		6	3	3	22
4	Inverte-brate	*Teredo navalis (*Linnaeus, 1758)	x				x		x			10		6	3	3	22
5	Phyto	*Karenia mikimotoi* (G. Hansen & Moestrup, 2000)	x	x	x				x			10		6	2	3	21
6	Fish	*Oncorhynchus mykiss* (Walbaum, 1792)	x						x			10		4	3	4	21
7	Zoo-plankton	*Mnemiopsis leidyi (*A. Agassiz, 1865)	x		x		x		x			10		6	3	2	21
8	Inverte-brate	*Eriocheir sinensis (* H. Milne Edwards, 1853	x		x		x		x			10		4	3	3	20
9	Inverte-brate	*Rhithropanopeus harrisii* (Gould, 1841)	x	x	x				x			10		4	3	3	20
10	Phyto	*Prorocentrum minimum* (Pavillard) (Schiller, 1933)	x						x			10		4	2	3	19
11	Sub. Veg.	*Gracilaria vermiculophylla* (Ohmi) (Papenfuss, 1967)	x	x	x		x		x			10		6	2	1	19
12	Sub. Veg.	*Sargassum muticum* (Yendo) (Fensholt, 1955)	x			x	x		x			10		6	2	1	19
13	Inverte-brate	*Cordylophora caspia* (Pallas, 1771)	x						x			10		4	2	3	19
14	Inverte-brate	*Styela clava* (Herdman, 1881)	x	x					x			10		4	2	3	19
15	Inverte-brate	*Dreissena polymorpha* (Pallas, 1771)		x	x		x		x			8		6	2	3	19
16	Fish	*Acipenser baerii*									x	8		4	3	4	19
17	Fish	*Acipenser gueldenstaedtii*									x	8		4	3	4	19

Nr.	Group	Name	DCE	HELCOM	OSPAR	EU-forord-ning	NST 1	NST 2	KJ 1	KJ 2	PRM	Criteria 1 established NIS	Criteria 2 potential NIS	Criteria 3 invasive species	Criteria 4 ease of deter-mination	Criteria 5 eDNA	Score
18	Fish	*Acipenser ruthenus*									x	8		4	3	4	19
19	Fish	*Acipenser stellatus*									x	8		4	3	4	19
20	Fish	*Huso huso*									x	8		4	3	4	19
21	Fish	*Oncorhynchus gorbuscha*									x	8		4	3	4	19
22	Fish	*Salvelinus fontinalis* (Mitchill, 1814)							x			8		4	3	4	19
23	Sub. Veg.	*Colpomenia peregrina (*Sauvageau, 1927)	x					x	x			10		4	3	1	18
24	Inverte-brate	*Ensis Americanus (Gould)*	x	x	x		x		x			8		4	3	3	18
25	Sub. Veg.	*Dasya baillouviana* (Gmelin) (Montagne, 1841)	x					x	x			10		4	2	1	17
26	Sub. Veg.	*Heterosiphonia japonica (*Yendo, 1920)	x					x	x			10		4	2	1	17
27	Sub. Veg.	*Spartina angelica*	x				x		x			10		4	2	1	17
28	Phyto	*Heterosigma akashiwo*	x									8		4	2	3	17
29	Phyto	*Pseudochattonella farcimen* (W. Eichrem, 2009)							x			8		4	2	3	17
30	Invert-brate	*Molgula manhattensis* (de Kay, 1843)							x			8		4	2	3	17
31	Zoo-plankton	*Cercopagis pengoi* (Ostroumov, 1891)		x	x			x		x		0	6	6	3	2	17
32	Inverte-brate	*Homarus americanus (*H. Milne Edwards, 1837)				x		x		x		0	5	6	3	3	17
33	Inverte-brate	*Paralithodes camtschaticus* (Tilesius, 1815)			x			x		x		0	5	6	3	3	17
34	Inverte-brate	*Didemnum vexillum (*Kott, 2002)		x	x	x				x		0	8	4	2	3	17
35	Sub. Veg.	*Fucus evanescens* C. (Agardh, 1820)	x						x			10		4	1	1	16
36	Inverte-brate	*Petricolaria pholadiformis* (Lamarck, 1818)	x						x			10		0	3	3	16
-	Parasites	*Anguillicola crassus* Kuwahara, (Niimi & Itagaki, 1974)	x				x		x			10		4	1	1	16

Nr.	Group	Name	DCE	HELCOM	OSPAR	EU-forord-ning	NST 1	NST 2	KJ 1	KJ 2	PRM	Criteria 1 established NIS	Criteria 2 potential NIS	Criteria 3 invasive species	Criteria 4 ease of deter-mination	Criteria 5 eDNA	Score
37	Fish	*Perccottus glenii*				x							3	6	3	4	16
38	Inverte-brate	*Elminius modestus (*Darwin, 1854)	x		x				x			10		0	2	3	15
39	Invert-brate	*Ficopomatus enigmaticus* (Fauvel, 1923)	x	x	x				x			10		0	2	3	15
40	Inverte-brate	*Marenzelleria viridis* (Verrill, 1873)	x		x				x			10		0	2	3	15
41	Inverte-brate	*Ocenebra inornata* (Récluz, 1851)	x						x			10		0	2	3	15
42	Inverte-brate	*Potamopyrgus antipodarum* (J.E. Gray, 1843)	x						x			10		0	2	3	15
43	Phyto	*Pseudochattonella verriculosum*	x	x	x							10		0	2	3	15
44	Sub. Veg.	*Codium fragile* subsp. *fragile* (Suringar) (Hariot, 1889)						x	x			8		4	2	1	15
45	Fish	*Carassius auratus auratus* (Linnaeus, 1758)					x		x			8		0	3	4	15
46	Fish	*Cyprinus carpio (*Linnaeus, 1758)						x	x			8		0	3	4	15
47	Inverte-brate	*Mya arenaria (*Linnaeus, 1758)	x						x			10		0	2	2	14
48	Zoo-plankton	*Penilia avirostris (*Dana, 1849)	x						x			10		0	2	2	14
49	Inverte-brate	*Diadumene lineata* (Verrill, 1869)							x			8		0	3	3	14
50	Sub. Veg.	*Bonnemaisonia hamifera (*Hariot, 1891)	x					x	x			10		0	2	1	13
51	Phyto	Alexandrium catenella		x	x							8		0	2	3	13
52	Phyto	Alexandrium minutum (Halim, 1960)							x			8		0	2	3	13
53	Phyto	Alexandrium tamarense (Lebour) Balech, 1995							x			8		0	2	3	13
54	Phyto	Coscinodiscus wailesii (Gran & Angst, 1931)		x	x				x			8		0	2	3	13
55	Phyto	Gymnodinium chlorophorum	x									8		0	2	3	13

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56	Phyto	Odontella sinensis (Greville) (Grunow, 1884)							x			8		0	2	3	13
57	Inverte-brate	Balamus impovisus (Darwin)	x									8		0	2	3	13
58	Inverte-brate	Caprella mutica (Schurin, 1935)		x	x	x			x			8		0	2	3	13
59	Inverte-brate	Caulleriella killariensis (Southern, 1914)							x			8		0	2	3	13
60	Inverte-brate	Neanthes succinea (Frey & Lauckart)	x									8		0	2	3	13
61	Inverte-brate	Pachycordyle navis (Millard, 1959)							x			8		0	2	3	13
62	Inverte-brate	Platorchestia platensis (Krøyer, 1845)							x			8		0	2	3	13
63	Inverte-brate	Telmatogeton japonicus (Tokunaga, 1933)							x			8		0	2	3	13
64	Inverte-brate	Limulus polyphemus (Linnaeus, 1758)								x		0	3	4	3	3	13
65	Inverte-brate	Gammarus tigrinus (Sexton, 1939)		x	x					x		0	4	4	2	3	13
66	Zoo-plankton	Acartia tonsa (Dana, 1849)	x		x				x			10		0		2	12
67	Sub. Veg.	Neosiphonia harveyi (J.W. Bailey) (M.-S. Kim, H.-G. Choi, Guiry & G.W. Saunders, 2001)	x						x			10		0	1	1	12
68	Parasites	Gonionemus vertens (A. Agassiz, 1862)	x						x			10		0	1	1	12
69	Parasites	Pseudodactylogyrus anguillae (Yin & Sproston, 1948)	x				x		x			10		0	1	1	12
70	Inverte-brate	Rapana venosa (Valenciennes, 1846)		x	x	x		x		x		0	7	0	2	3	12
71	Parasites	Pseudodactylogyrus bini (Kikuchi, 1929)	x						x			10		0		1	11
72	Sub. Veg.	Aglaothamnion halliae (F.S. Collins) (N.E. Aponte, D.L. Ballantine & J.N. Norris, 1997)	x							x		8		0	2	1	11

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73	Sub. Veg.	Dictuota dichotoma (Hudson) (J.V. Lamoutoux)	x									8		0	2	1	11
74	Sub. Veg.	Porphyra umbilicalis (Kützing, 1843)							x			8		0	2	1	11
75	Inverte-brate	Callinectes sapidus (Rathbun, 1896)		x	x			x		x		0	5	0	3	3	11
76	Inverte-brate	Corbicula fluminea (O.F. Müller, 1774)		x	x					x		0	5	0	3	3	11
77	Inverte-brate	Hydroides dianthus		x	x							0	6	0	2	3	11
78	Inverte-brate	Mytilopsis leucophaeta (Conrad, 1831)		x	x					x		0	5	0	3	3	11
79	Sub. Veg.	Codium fragile subsp. Tomentosoides (van Goor) (P.C. Silva)	x									8		0	1	1	10
80	Zoo-plankton	Hemimysis anomala (G.O. Sars, 1907)		x	x					x		0	6	0	2	2	10
81	Inverte-brate	Hemigrapsus sanguinus (de Haan, 1835)		x	x					x		0	5	0	2	3	10
82	Inverte-brate	Hemigrapsus takanoi (Asakura & Watanabe, 2005)		x	x					x		0	5	0	2	3	10
83	Inverte-brate	Marenzelleria neglecta (Sikorski & Bick, 2004)		x	x					x		0	5	0	2	3	10
84	Parasites	Aphanomyces astaci Schikora, 1906					x		x			8		0		1	9
85	Inverte-brate	Dikerogammarus villosus (Sowinsky, 1894)		x	x					x		0	4	0	2	3	9
86	Inverte-brate	Venerupis philippinarum (A. Adams & Reeve, 1850)								x		0	3	0	3	3	9
87	Fish	Gambusia holbrooki (Girard, 1859)								x		0	2	0	3	4	9
88	Phyto	Alexandrium leei (Balech, 1985)								x		0	3	0	2	3	8
89	Phyto	Gymnodinium catenatum (L.W. Graham, 1943)								x		0	3	0	2	3	8

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90	Zoo-plankton	Beroe ovata (Bruguière, 1789)								x		0	3	0	3	2	8
91	Inverte-brate	Asterias amurensis		x	x							0	2	0	3	3	8
92	Inverte-brate	Chelicorophium curvispinum (G.O: Sars, 1895)								x		0	3	0	2	3	8
93	Inverte-brate	Gonionemus vertens (A. Agassiz, 1862)								x		0	2	0	3	3	8
94	Inverte-brate	Mercenaria mercenaria (Linnaeus, 1758)								x		0	3	0	2	3	8
95	Inverte-brate	Potamothrix heuscheri (Bretscher, 1900)								x		0	3	0	2	3	8
96	Phyto	Dinophysis sacculus		x	x							0	2	0	2	3	7
97	Phyto	Fibrocapsa japonica		x	x							0	2	0	2	3	7
98	Phyto	Pfiesteria piscicida		x	x							0	2	0	2	3	7
99	Zoo-plankton	Evadne anonyx (G.O. Sars, 1897)								x		0	3	0	2	2	7
100	Zoo-plankton	Limnomysis benedeni (Czerniavsky, 1882)								x		0	3	0	2	2	7
101	Inverte-brate	Anadara transversa			x							0	1	0	3	3	7
102	Inverte-brate	Arcuatula senhousia		x	x							0	2	0	2	3	7
103	Inverte-brate	Botrylloides violaceus (Oka, 1927)								x		0	3	0	1	3	7
104	Inverte-brate	Bugula neritina (Linnaeus, 1758)								x		0	3	0	1	3	7
105	Inverte-brate	Chama pacifica			x							0	1	0	3	3	7
106	Inverte-brate	Chionoecetes opilio			x							0	1	0	3	3	7
107	Inverte-brate	Corella eumyota (Traustedt, 1882)								x		0	2	0	2	3	7
108	Inverte-brate	Dikerogammarus haemobaphes (Eichwald, 1841)								x		0	2	0	2	3	7

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109	Inverte-brate	Dreissena bugensis (Andrusov, 1897)		x	x					x		0	2	0	2	3	7
110	Inverte-brate	Gmelinoides fasciatus (Stebbing, 1899)								x		0	2	0	2	3	7
111	Inverte-brate	Monocorophium sextonae (Crawford, 1937)								x		0	2	0	2	3	7
112	Inverte-brate	Mytilus galloprovincialis		x	x							0	2	0	2	3	7
113	Inverte-brate	Palaemon macrodactylus		x	x							0	2	0	2	3	7
114	Inverte-brate	Perophora japonica (Oka, 1927)								x		0	2	0	2	3	7
115	Inverte-brate	Pontogammarus robustoides (G.O. Sars, 1894)								x		0	2	0	2	3	7
116	Inverte-brate	Potamocorbula amurensis		x	x							0	2	0	2	3	7
117	Inverte-brate	Rangia cuneata		x	x							0	2	0	2	3	7
118	Fish	Neogobius fluviatilis (Pallas, 1814)								x		0	0	0	3	4	7
119	Fish	Neogobius gymnotrachelus (Kessler, 1857)								x		0	0	0	3	4	7
120	Fish	Neogobius kessleri, (Günther,1861)								x		0	0	0	3	4	7
121	Fish	Proterorhinus marmoratus,(Pallas, 1814)								x		0	0	0	3	4	7
122	Phyto	Alexandrium monilatum			x							0	1	0	2	3	6
123	Phyto	Alexandrium ostenfeldii		x								0	1	0	2	3	6
124	Phyto	Phaeocystis pouchetii			x							0	1	0	2	3	6
125	Sub. Veg.	Undaria pinnatifida (Harvey) (Suringar, 1873)		x	x					x		0	3	0	2	1	6
126	Inverte-brate	Amphibalanus eburneus, ex. Balanus eburnus			x							0	1	0	2	3	6
127	Inverte-brate	Brachidontes pharaonis			x							0	1	0	2	3	6

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128	Inverte-brate	Hydroides elegans			x							0	1	0	2	3	6
129	Inverte-brate	Microcosmus squamiger			x							0	1	0	2	3	6
130	Inverte-brate	Nematostella vectensis Stephenson, 1935								x		0	0	0	3	3	6
131	Inverte-brate	Nemopsis bachei L. Agassiz, 1849								x		0	0	0	3	3	6
132	Inverte-brate	Palaemon elegans		x								0	1	0	2	3	6
133	Inverte-brate	Tricellaria inopinata d'Hondt & Occhipinti Ambrogi, 1985								x		0	2	0	1	3	6
134	Inverte-brate	Urosalpinx cinerea (Say, 1822)								x		0	1	0	2	3	6
135	Zoo-plankton	Maeotias marginata (Modeer, 1791)								x		0	0	0	3	2	5
136	Zoo-plankton	Mertensia ovum (Fabricius, 1780)								x		0	0	0	3	2	5
137	Sub. Veg.	Caulerpa taxifolia			x							0	1	0	3	1	5
138	Sub. Veg.	Grateloupia turuturu		x	x							0	2	0	2	1	5
139	Inverte-brate	Branchiura sowerbyi Beddard, 1892								x		0	0	0	2	3	5
140	Inverte-brate	Diadumene cincta Stephenson, 1925								x		0	0	0	2	3	5
141	Inverte-brate	Incisocalliope aestuarius (Watling & Maurer, 1973)								x		0	0	0	2	3	5
142	Inverte-brate	Marenzelleria arctica (Chamberlin, 1920)								x		0	0	0	2	3	5
143	Inverte-brate	Obesogammarus crassus (G.O. Sars, 1894)								x		0	0	0	2	3	5
144	Inverte-brate	Paranais frici Hrabe, 1941								x		0	0	0	2	3	5
145	Inverte-brate	Potamothrix bavaricus (Oschmann, 1913)								x		0	0	0	2	3	5

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146	Inverte-brate	Potamothrix bedoti (Piguet, 1913)								x		0	0	0	2	3	5
147	Inverte-brate	Potamothrix moldaviensis (Vejdovsky & Mrázek, 1903)								x		0	0	0	2	3	5
148	Inverte-brate	Potamothrix vejdovskyi (Hrabe, 1941)								x		0	0	0	2	3	5
149	Sub. Veg.	Grateloupia doryphore				x						0	1	0	2	1	4
150	Sub. Veg.	Halophila stipulacea				x						0	1	0	2	1	4
151	Sub. Veg.	Lophocladia lallemandii				x						0	1	0	2	1	4
152	Sub. Veg.	Stypopodium schimperi				x						0	1	0	2	1	4
153	Inverte-brate	Ameira divagans (Nicholls, 1939)								x		0	0	0	1	3	4
154	Inverte-brate	Garveia franciscana (Torrey, 1902)								x		0	0	0	1	3	4
155	Inverte-brate	Jaera (Jaera) sarsi (Vakkanov, 1936)								x		0	0	0	1	3	4
156	Inverte-brate	Victorella pavida (Saville Kent, 1870)								x		0	0	0	1	3	4
157	Zoo-plankton	Paramysis (Mesomysis) intermedia (Czerniavsky, 1882)								x		0	0	0	1	2	3
158	Zoo-plankton	Paramysis (Serrapalpisis) lacustris (Czerniavsky, 1882)								x		0	0	0	1	2	3
159	Sub. Veg.	Caulerpa cylindracea				x						0	1	0	1	1	3
160	Parasites	Bonamia ostreae Pichot (Comps, Tigé, Grizel & Rabouin, 1980)								x		0	1	0	1	1	3
161	Parasites	Marteilia refringens (Grizel, Comps, Bonami, Cousserans, Duthoit & Le Pennec, 1974)								x		0	1	0	1	1	3
162	Parasites	Myicola ostreae (Hoshina & Sugiura, 1953)								x		0	1	0	1	1	3
163	Parasites	Mytilicola orientalis (Mori, 1935)								x		0	1	0	1	1	3
164	Parasites	Pseudobacciger harengulae (Yamaguti, 1938)								x		0	1	0	1	1	3

NIVA Denmark is the name, water is our game

NIVA Denmark Water Research is a regional office of the Norwegian Institute for Water Research (NIVA) and has just recently been established to resolve environmental issues concerning the freshwater and marine systems that relate to Denmark.

NIVA Denmark has primary focus on research-based implementation of a number of EU's directives *inter alia* the Water Framework Directive and the Marine Strategy Framework Directive together with international conventions (HELCOM, OSPAR, BDC). We occasionally provide consultancy to authorities and small and medium-sized companies.

NIVA Denmark is a a place for practice, observation, testing and synthesis. Key research and test areas include eutrophication, hazardous substances, biodiversity, and ecosystem health as well as the implications of multiple human activities in marine waters and in streams, rivers and lakes. We develop indicators, monitoring methods and tools to assess the state of an ecosystem in order to carry out analyses and contribute to evidence based and sustainable solutions to the challenges we and the environment face.

NIVA Denmark, as a regional office to NIVA has thus the backing of more than 200 dedicated researchers and experts.

Winghouse
Ørestads Boulevard 73
2300 Copenhagen S
Denmark
Telephone: +45 88 96 96 70
E-mail: post@niva-denmark.dk
CVR/VAT no.: 35431063
www.niva-denmark.dk